DOCUMENT RESUME

ED 224 892 CE 034 547

TITLE	Medical Laboratory Technician--Microbiology, 10-3. Military Curriculum Materials for Vocational and Technical Education.
INSTITUTION	Air Force Training Command, Sheppard AFB, Tex.; Ohio State Univ., Columbus. National Center for Research in Vocational Education.
SPONS AGENCY	Office of Education (DHEW), Washington, D.C.
PUB DATE	78
NOTE	600p.; For related documents see CE 034 546-548.
PUB TYPE	Guides - Classroom Use - Materials (For Learner) (051)
EDRS PRICE	MF03/PC24 Plus Postage.
DESCRIPTORS	Allied Health Occupations Education; Behavioral Objectives; Chemical Analysis; Chemistry; Correspondence Study; *Culturing Techniques; Independent Study; *Job Skills; *Laboratory Procedures; Laboratory Technology; Learning Modules; Medical Education; *Medical Laboratory Assistants; *Microbiology; Postsecondary Education; Secondary Education; *Units of Study; Vocational Education
IDENTIFIERS	Military Curriculum Project

ABSTRACT

This course, the second of three courses in the medical laboratory technician field adapted from military curriculum materials for use in vocational and technical education, was designed as a refresher course for student self-study and evaluation. It is suitable for use by advanced students or beginning students participating in a supervised laboratory or on-the-job learning situation. The course is divided into three volumes containing student workbooks, readings, and tests. Volume 1 covers blood composition and functions, blood counts, ertrocytes, leukocytes, and coagulation. Volume 2 presents information concerning blood banking; this information includes immunohematology, blood group systems, transfusion of blood, and the operation of a blood center. The final volume discusses the principles of serology, the agglutination test, the fixation and precipitin test, and the serological test for syphilis. A glossary of technical terms used in the three volumes is printed at the back of volume 3. Each of the volumes contains chapters with objectives, text, review exercises, and answers to the exercises. A volume review exercise (without answers) is provided. (KC)

****
* Reproductions supplied by EDRS are the best that can be made *
* from the original document. *
****

ED224892

U.S. DEPARTMENT OF EDUCATION
NATIONAL INSTITUTE OF EDUCATION
EDUCATIONAL RESOURCES INFORMATION
CENTER (ERIC)

☑ This document has been reproduced as received from the person or organization originating it.

☐ Minor changes have been made to improve reproduction quality.

● Points of view or opinions stated in this document do not necessarily represent official NIE position or policy.

"PERMISSION TO REPRODUCE THIS
MATERIAL HAS BEEN GRANTED BY

TO THE EDUCATIONAL RESOURCES
INFORMATION CENTER (ERIC)."

MILITARY CURRICULUM MATERIALS

The military-developed curriculum materials in this course package were selected by the National Center for Research in Vocational Education Military Curriculum Project for dissemination to the six regional Curriculum Coordination Centers and other instructional materials agencies. The purpose of disseminating these courses was to make curriculum materials developed by the military more accessible to vocational educators in the civilian setting.

The course materials were acquired, evaluated by project staff and practitioners in the field, and prepared for dissemination. Materials which were specific to the military were deleted, copyrighted materials were either omitted or approval for their use was obtained. These course packages contain curriculum resource materials which can be adapted to support vocational instruction and curriculum development.

The National Center Mission Statement

The National Center for Research in Vocational Education's mission is to increase the ability of diverse agencies, institutions, and organizations to solve educational problems relating to individual career planning, preparation, and progression. The National Center fulfills its mission by:

- Generating knowledge through research
- Developing educational programs and products
- Evaluating individual program needs and outcomes
- Installing educational programs and products
- Operating information systems and services
- Conducting leadership development and training programs

FOR FURTHER INFORMATION ABOUT
Military Curriculum Materials
WRITE OR CALL
Program Information Office
The National Center for Research in Vocational Education
The Ohio State University
1960 Kenny Road, Columbus, Ohio 43210
Telephone: 614/486-3655 or Toll Free 800/848-4815 within the continental U.S. (except Ohio)

THE NATIONAL CENTER
FOR RESEARCH IN VOCATIONAL EDUCATION
The Ohio State University · 1960 Kenny Road · Columbus, Ohio 43210
Tel (614) 486-3655 Cable CTVOCEDOSU/Columbus, Ohio

Military Curriculum Materials for Vocational and Technical Education

Information and Field
Services Division

The National Center for Research
in Vocational Education

Military Curriculum Materials Dissemination Is . . .

an activity to increase the accessibility of military developed curriculum materials to vocational and technical educators.

This project, funded by the U.S. Office of Education, includes the identification and acquisition of curriculum materials in print form from the Coast Guard, Air Force, Army, Marine Corps and Navy.

Access to military curriculum materials is provided through a "Joint Memorandum of Understanding" between the U.S. Office of Education and the Department of Defense.

The acquired materials are reviewed by staff and subject matter specialists, and courses deemed applicable to vocational and technical education are selected for dissemination.

The National Center for Research in Vocational Education is the U.S. Office of Education's designated representative to acquire the materials and conduct the project activities.

Project Staff:

Wesley E. Budke, Ph.D., Director
National Center Clearinghouse

Shirley A. Chase, Ph.D.
Project Director

What Materials Are Available?

One hundred twenty courses on microfiche (thirteen in paper form) and descriptions of each have been provided to the vocational Curriculum Coordination Centers and other instructional materials agencies for dissemination.

Course materials include programmed instruction, curriculum outlines, instructor guides, student workbooks and technical manuals.

The 120 courses represent the following sixteen vocational subject areas:

- Agriculture
- Aviation
- Building & Construction Trades
- Clerical Occupations
- Communications
- Drafting
- Electronics
- Engine Mechanics
- Food Service
- Health
- Heating & Air Conditioning
- Machine Shop
- Management & Supervision
- Meteorology & Navigation
- Photography
- Public Service

The number of courses and the subject areas represented will expand as additional materials with application to vocational and technical education are identified and selected for dissemination.

How Can These Materials Be Obtained?

Contact the Curriculum Coordination Center in your region for information on obtaining materials (e.g., availability and cost). They will respond to your request directly or refer you to an instructional materials agency closer to you.

CURRICULUM COORDINATION CENTERS

EAST CENTRAL
Rebecca S. Douglass
Director
100 North First Street
Springfield, IL 62777
217/782-0759

NORTHWEST
William Daniels
Director
Building 17
Airdustrial Park
Olympia, WA 98504
206/753-0879

MIDWEST
Robert Patton
Director
1515 West Sixth Ave.
Stillwater, OK 74704
405/377-2000

SOUTHEAST
James F. Shill, Ph.D.
Director
Mississippi State University
Drawer DX
Mississippi State, MS 39762
601/325-2510

NORTHEAST
Joseph F. Kelly, Ph.D.
Director
225 West State Street
Trenton, NJ 08625
609/292-6562

WESTERN
Lawrence F. H. Zane, Ph.D.
Director
1776 University Ave.
Honolulu, HI 96822
808/948-7834

MEDICAL LABORATORY TECHNICIAN - MICROBIOLOGY

Table of Contents

Course Description — Page 1

Volume 1

Laboratory Procedures in Clinical Bacteriology (Part I) - Text Material — Page 3
Laboratory Procedures in Clinical Bacteriology (Part I) - Workbook — Page 112

Volume 2

Laboratory Procedures in Clinical Bacteriology (Part II) - Text Material — Page 174
Laboratory Procedures in Clinical Bacteriology (Part II) - Workbook — Page 245
Supplementary Material — Page 293

Volume 3

Clinical Parasitology - Text Material — Page 306
Clinical Parasitology - Workbook — Page 371

Volume 4

Laboratory Procedures in Clinical Mycology - Text Material — Page 415
Laboratory Procedures in Clinical Mycology - Workbook — Page 515

3

90412 01 0370 0973

CDC 90412

MEDICAL LABORATORY TECHNICIAN - MICROBIOLOGY

(AFSC 90470)

Volume 1

Laboratory Procedures in Clinical Bacteriology (Part I)

10-3

Extension Course Institute

Air University

MEDICAL LABORATORY TECHNICIAN–MICROBIOLOGY

Correspondence Course **10-3** 1

Developed by:

United States Air Force

Development and Review Date:

Unknown

Occupational Area:

Health

Cost: **Print Pages:**

Availability:
Military Curriculum Project, The Center for Vocational Education, 1960 Kenny Rd., Columbus, OH 43210

Suggested Background:

Chemistry, biology, zoology, completion of *Medical Laboratory Technician–Clinical Chemistry and Urinalysis*, (10-2)

Target Audiences:

Grades 10-adult

Organization of Materials:

Student workbooks containing objectives, assignments, chapter review exercises and answers, volume review exercises; texts; supplemental materials

Type of Instruction:

Individualized, self-paced

Type of Materials:		No. of Pages:	Average Completion Time:
Volume 1 –	*Laboratory Procedures in Clinical Bacteriology* (Part I)	100	Flexible
	Workbook	61	
Volume 2 –	*Laboratory Procedures in Clinical Bacteriology* (Part II)	67	Flexible
	Workbook	47	
	Supplementary Materials	8	
Volume 3 –	*Clinical Parasitology*	61	Flexible
	Workbook	42	
Volume 4 –	*Laboratory Procedures in Clinical Mycology*	[illegible]	Flexible
	Workbook	55	

Supplementary Materials Required:

None

THE CENTER FOR VOCATIONAL EDUCATION
THE OHIO STATE UNIVERSITY

Expires July 1, 1978

Course Description

This course is the second of three courses in the Medical Laboratory Technician field to upgrade the Specialist (skilled) worker to the Technician (advanced) level. The course contains basic information and could be used as a refresher course, but is designed to be used by advanced students or beginning students in a supervised laboratory or on-the-job learning situation. The first course, *Medical Laboratory Technician-Clinical Chemistry and Urinalysis*, is a prerequisite to this course. The remaining course is, *Hematology, Serology, Blood Banking, and Immunohematology*. This course lists the following duties for a Medical Laboratory Technician/Specialist:

- Performs hematological tests
- Performs urinalysis
- Performs chemical analysis
- Assists in blood bank duties
- Performs microbiological and serological tests
- Accomplishes general medical laboratory duties
- Supervises medical laboratory personnel

This course is divided into four volumes containing student workbooks, texts, and supplementary material.

Volume 1 — *Laboratory Procedures in Clinical Bacteriology (Part I)* traces the historical development of current bacteriological procedures, instructs on laboratory techniques and equipment necessary for the safe handling of infectious microorganisms, explains how knowledge of bacterial morphology and physiology permits the technician to isolate and cultivate pathogenic microbes, and discloses the basis for anti-microbial sensitivity testing and drug assays.

Volume 2 — *Laboratory Procedures in Clinical Bacteriology (Part II)* contains information on Gram-positive organisms, Gram-negative cocci and coccoid forms, the small Gram-negative bacilli, the enteric organisms, acid-fast bacilli, and spirochetes. Volume 2 is accompanied by a book of eight charts using a flow-chart format to identify organisms.

Volume 3 — *Clinical Parasitology* introduces medical parasitology, and explains protozoa infecting man, platyhelminthes infecting man and nematodes infecting man.

Volume 4 — *Laboratory Procedures in Clinical Mycology* discusses characteristics of fungi such as taxonomic relationships, cellular morphology, cultural properties, and the fungi as disease agents. Also covered are collection and processing techniques and how to ship specimens; yeast like fungi, monomorphic molds, dimorphic fungi, and saprophytic fungi; and guidelines for collection, preservation, packaging and shipment of clinical virological specimens.

Each of the volumes contains chapters with objectives, text, review exercises and answers to the exercises. A volume review exercise is provided but no answers are available. This course was designed for student self-study and evaluation within the context of a laboratory or on-the-job learning situation. The material is useful for beginning students with a good science and math background or workers who wish to upgrade or refresh their skills. Much of the material involves review of basic procedures. Some supervisory information is also included.

5

Preface

THIS SECOND course in a series covering the medical laboratory technician specialty is devoted to the principles and practices of clincial microbiology. CDC 90411, *Clinical Chemistry and Urinalysis*, served as a prerequisite. CDC 90413, which follows the course in Microbiology, will introduce the concepts underlying conventional laboratory procedures in immunohematology, serology and blood banking.

Volumes 1 and 2 of the course in microbiology take up the fundamentals of diagnostic bacteriology. In the third volume, the life processes and identifying features of intestinal, blood, and tissue parasites are explored. Volume 4 on mycology covers laboratory methods used in identifying pathogenic fungi. The last volume deals with the nature of the virus and rickettsial agents of disease.

The opening chapter of Volume 1 traces the historical development of current bacteriological procedures. The second chapter instructs on laboratory techniques and equipment necessary for the safe handling of infectious microorganisms. Chapters 3 and 4 explain how knowledge of bacterial morphology and physiology permits the technician to isolate and cultivate pathogenic microbes in the laboratory. Lastly, chapter 5 discloses the basis for antimicrobial sensitivity testing and drug assays. In Volume 2 of this course the medically important genera of bacteria will be described, and specific measures outlined for establishing their identity.

If you have questions on the accuracy or currency of the subject matter of this text, or recommendations for its improvement, send them to Technical Training Center (MSSTW), Sheppard AFB, Texas 76311.

If you have questions on course enrollment or administration, or on any of ECI's instructional aids (Your Key to Career Development, Study Reference Guides, Chapter Review Exercises, Volume Review Exercise, and Course Examination), consult your education officer, training officer, or NCO, as appropriate. If he can't answer your questions, send them to ECI, Gunter AFB, Alabama 36114, preferably on ECI Form 17, Student Request for Assistance.

This volume is valued at 36 hours (12 points).

Material in this volume is technically accurate, adequate, and current as of January 1969.

12

Acknowledgment

Table 3 and figure 8 were furnished through the courtesy of American Sterilizer Company, Erie, Pennsylvania. Figures 31 and 32 were furnished through the courtesy of Millispore Corporation, Bedford, Massachusetts.

LIST OF CHANGES

COURSE NO.
90412

EFFECTIVE DATE OF SHIPPING LIST
16 Mar 76

CAREER FIELDS, POLICIES, PROCEDURES AND EQUIPMENT CHANGE. ALSO ERRORS OCCASIONALLY GET INTO PRINT. THE FOLLOWING ITEMS UPDATE AND CORRECT YOUR COURSE MATERIALS. PLEASE MAKE THE INDICATED CHANGES.

1. CHANGES FOR THE TEXT: VOLUME 1

a. Page 4, para 3-2, line 3: Delete "(SF 514 series)."

b. Page 5, para 3-11, line 4: Delete "SF 514K, Bacteriology."

c. Page 12, para 4-32, line 5: Change "SF 514K, Bacteriology" to "request form."

d. Page 17, para 5-14a, line 1: Insert "is" between "This" and "the."

e. Page 21, col 1: At the end of the column add "Do not put rubber, plastics, or textiles in this type sterilizer. Some plastics give off toxic fumes when heated at high temperatures."

f. Page 26, para 5-56, line 10: Change the first "and" to "are."

g. Page 31, para 5-83, line 5: Change "inside the wrapper of the material" to "in the center of the package."

h. Page 89, col 1, footnote, line 1: Change "outbraek" to "outbreak is."

2. CHANGES FOR THE TEXT: VOLUME 2

a. Page 7, col 1, line 3: After "organism;" change to read "then we place a 0.02 - 0.04 unit . . ."

b. Page 13, para 4-11, line 13: After "positive," insert "except for C. haemolyticum,."

c. Page 18, para 8-5, line 3: Delete "spores."

3. CHANGES FOR THE TEXT: VOLUME 3

a. Page 7, col 1, figure 1: Change "BLEPHAROPLAST" to "KINETOPLAST."

b. Page 9, col 1, figure 3: Change "BLEPHAROPLAST" to "KINETOPLAST."

4. CHANGE FOR THE TEXT: VOLUME 4

Page 46, col 2, figure 25: Insert "Coccidioides" between "of" and "immitis."

5. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 1

a. Page 4, Chapter Review Exercises, question 1 and 4: Delete "514."

b. Page 12, Chapter Review Exercises, question 56: Insert "70%" between "to" and "ethyl."

8

LIST OF CHANGES

COURSE NO. 90412	CAREER FIELDS, POLICIES, PROCEDURES AND EQUIPMENT CHANGE. ALSO ERRORS OCCASIONALLY GET INTO PRINT. THE FOLLOWING ITEMS UPDATE AND CORRECT YOUR COURSE MATERIALS. PLEASE MAKE THE INDICATED CHANGES.
EFFECTIVE DATE OF SHIPPING LIST 16 Mar 76	

5. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 1 (Continued)

c. Page 35, Chapter Review Exercises, chap 2, answer 1: Change "SF 514 series" to "the laboratory request forms."

d. Page 29, Chapter Review Exercises, question 45: Capitalize "hiss."

e. Page 39, Chapter Review Exercises, answer 56, line 1: Change "can be" to "when."

f. Page 42, Chapter Review Exercises, answer 46, line 1: Change "contamination" to "contaminating."

g. Page 43, Chapter Review Exercises, answer 13, line 1: Change "strongly recommended" to "mandatory."

h. Page 46, Chapter Review Exercises, answer 16: Change "PSA" to "PAS."

i. Page 47, Chapter Review Exercises, after answer 29, add: "30. Antibiotic sensitivity testing detects bacterial sensitivity (or resistance) to an antibiotic. Antibiotic assay determines the amount of the antibiotic in a specimen. (16-21)"

j. Page 56: Chapter 4 should begin with question 65 instead of question 64.

k. The following questions are no longer scored and need not be answered: 7, 69 and 82.

6. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 2

a. Page 5, Chapter Review Exercises, question 24, line 1: Change "The Bacitracin" to "The bacitracin." Change "to Bacitracis" to "to bacitracin."

b. Page 7, Chapter Review Exercises, question 37: Change "corynebacterium" to "corynebacteria."

c. Page 20, Chapter Review Exercises, question 13, line 1: Delete "and Escherichia coli,."

d. Page 23, Chapter Review Exercises, question 36: Change "Morphological" to "Morphologically,." Chapter 5, Objectives, line 2: Change "biomedical" to "biochemical."

e. Page 33, Chapter Review Exercises, answer 15: Change "Anaerobes" to "The Veillonellae are anaerobes."

f. Page 42, question 26, choice a. Change the word "strain" to "stain."

LIST OF CHANGES

COURSE NO. 90412

EFFECTIVE DATE OF SHIPPING LIST 16 Mar 76

CAREER FIELDS, POLICIES, PROCEDURES AND EQUIPMENT CHANGE. ALSO ERRORS OCCASIONALLY GET INTO PRINT. THE FOLLOWING ITEMS UPDATE AND CORRECT YOUR COURSE MATERIALS. PLEASE MAKE THE INDICATED CHANGES.

6. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 2 (Continued)

g. Page 43, question 30: In the stem of this question, change "veneral" to "venereal." Choice d: Change "veneral" to "venereal."

h. The following questions are no longer scored and need not be answered: 37 and 62.

7. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 3

a. Page 2, Chapter Review Exercises, Chapter 1, Objective, line 2: Change "parasitic" to "parasite."

b. Page 5, Chapter Review Exercises, question 8: Delete "or Rhodesian."

c. Page 5, Chapter Review Exercises, question 13: After "*Chilomastix mesnili*" add "cysts." Question 15: Delete "the parasite . . . of man" and substitute "*Giardia lamblia*."

d. Page 8, Chapter Review Exercises, question 27: Change "amoeba" to "amebae." Delete "quickly ruptures . . . tap water" and substitute "has no known cystic stage."

e. Page 9, Chapter Review Exercises, question 34: Change "merozoite" to "exoerythrocytic."

f. Page 12, Chapter Review Exercises, question 11, line 1: Delete "adult."

g. Page 13, Chapter Review Exercises, question 15, lines 1 and 2: Change "if you have . . . the eggs of" to "to determine whether the eggs were produced by" and after "*Fasciola hepatica*" change "from those of" to "or." Question 20: Change "the species . . . in exercise 11" to "*Fasciolopsis buski*."

h. Page 14, Chapter Review Exercises, question 21: Change "the species . . . exercise 11" to "*F. Buski*."

i. Page 15, Chapter Review Exercises, question 33: Change reference "(8-10)" to "(8-11)."

j. Page 17, Chapter Review Exercises, question 43: Change "cysticercus larva" to "*Taenia sp*. eggs."

k. Page 23, Chapter Review Exercises, question 30: Change "*ceylonicum*" to "*ceylanicum*."

10

LIST OF CHANGES

COURSE NO. 30412	CAREER FIELDS, POLICIES, PROCEDURES AND EQUIPMENT CHANGE. ALSO ERRORS OCCASIONALLY GET INTO PRINT. THE FOLLOWING ITEMS UPDATE AND CORRECT YOUR COURSE MATERIALS. PLEASE MAKE THE INDICATED CHANGES.
EFFECTIVE DATE OF SHIPPING LIST 16 Mar 76	

7. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 3 (Continued)

l. Page 30, Chapter Review Exercises, answer 23: Change "melacercariae" to "metacercariae." Answer 26: Delete "hooked." Answer 33: Change reference "(8-10)" to "(8-11)."

m. Page 31, Chapter Review Exercises, answer 1: Change "Nematodo" to "Nematoda."

n. Question 40 is no longer scored and need not be answered.

8. CHANGES FOR THE VOLUME WORKBOOK: VOLUME 4

a. Cover, title: Change "Micology" to "Mycology."

b. Page 10, Chapter Review Exercises, question 27: Change to read "Cladosporium werneckii causes what disease?"

c. Page 28, Chapter Review Exercises, question 17, line 2: Change "enter tracts" to "intestinal tracts."

d. Page 36, Chapter Review Exercises, question 68: Change "labated" to "lobated."

e. Page 40, Chapter Review Exercises, answer 16: Change "polio and" to "polio,." Answer 18, line 1: Change "encephalitci" to "encephalitic."

f. Page 41, Chapter Review Exercises, answer 32, line 1: Change "Senological" to "Serological."

g. Page 44, question 5: Change guide number "(400)" to "(401)."

h. Page 45, question 18 should be in Chapter 2 instead of Chapter 1. Question 19, choice b: Change "Tecture" to "Texture."

i. Page 49, question 56: In the stem of the question, change "Histoplasmosis" to "Histoplasma."

j. Page 51, question 69, choice a: Delete "capsulatum."

k. The following questions are no longer scored and need not be answered: 9, 27, 79 and 80.

NOTE: Change the currency date on all volumes to "December 1974."

Contents

Page

Preface iii

Acknowledgment iv

Chapter

1 HISTORY OF BACTERIOLOGY 1

2 ASEPTIC TECHNIQUES AND STERILIZATION PROCEDURES 4

3 BACTERIAL MORPHOLOGY AND PHYSIOLOGY 33

4 INOCULATION OF MEDIA FOR ISOLATION OF BACTERIA 59

5 ANTIMICROBIAL AGENTS, DRUG RESISTANCE AND SENSITIVITY TESTING 83

Bibliography 97

Glossary 99

CHAPTER 1

History of Bacteriology

BACTERIOLOGY, as we know it today, is a science which demands an understanding of the basic morphology and physiology of bacteria. You must have a keen sense of perception to be able to observe bacterial growth, make intelligent interpretations of this growth, and record what you observe. As a laboratory technician working in bacteriology, you must be a "detective" ready to analyze clues gathered from your efforts in the laboratory as to the identity of a particular disease-associated microorganism. This analysis is fairly routine today, but it has not always been this way.

2. Sanitary practices and devices to improve ventilation and dispose of human wastes were in use as far back in history as the Knossian or Minoan period (1850–1400 B.C.). Some of these sanitary methods were far better than anything in use before the 19th century. There were no bacteriologists during this time period, so we must conclude that aloof, intelligent, logical minds prevailed in the hierarchy of the day.

3. Investigators of long ago were not aware of anything smaller than what was visible to the unaided eye. Belief in the supernatural, fear of reprisal, and religious practices did not help to stimulate research. The invention of the microscope was an important stimulus to learn about the tiny world of *animalcules*. Through a slow and gradual process, knowledge about bacteria was sought and diseases associated with them were documented. The world began to realize that small microscopic matter was important in health and disease.

4. Recent history of medical bacteriology is largely that of the foundation of techniques for isolation and identification of specific disease-causing microorganisms. Conscientious experimentation and careful recording of observations have led to the present status of bacteriology as a science and as a useful tool in fighting the diseases of man.

5. By being aware of some of the historical high points in the development of bacteriology as a science, you will better appreciate the vast obstacles which had to be overcome. Hopefully, you will be stimulated to add to the progress in this science. Before we go into our discussion of present-day laboratory techniques, we will look at some of the events which helped to shape the destiny of bacteriology.

1. Major Contributions in the Discovery of Bacteria

1-1. Hippocrates (460–377 B.C.), the father of medicine, was not a bacteriologist, but his observations and writings gave a foundation to the field of bacteriology, even though bacteria as such were still unknown. He attributed diseases to changes and disorders in the "vital fluids" of the body. He stressed the use of boiled water for irrigating wounds and called attention to the importance of clean hands and fingernails of the "surgeon." He coined such terms as "acute," "chronic," "endemic," and "epidemic"; terms which are still with us today although we may use them in a slightly different context.

1-2. As the Dark Ages replaced the progressiveness of the Romans and Greeks with mysticism and fear, witches were boiled instead of water. Filth, pestilence, and plague covered Europe. This situation prevailed until the 18th century. Changes were made, but their acceptance was slow.

1-3. It was during the Renaissance (1453–1600) that bacteriology probably had its real beginning. In 1546 Girolamo Fracastoro, father of the germ theory of disease wrote, "Contagion is an infection that passes from one thing to another." He recognized that basically there were three sources of contagious material: (1) by contact, (2) by fomites, and (3) from a distance. This typified the level of scientific knowledge at the beginning of the 17th century, and from this level, science began to emerge as a systematic method of investigation. It was Fracastoro who should probably get credit for giving the venereal disease "syphilis" its name.

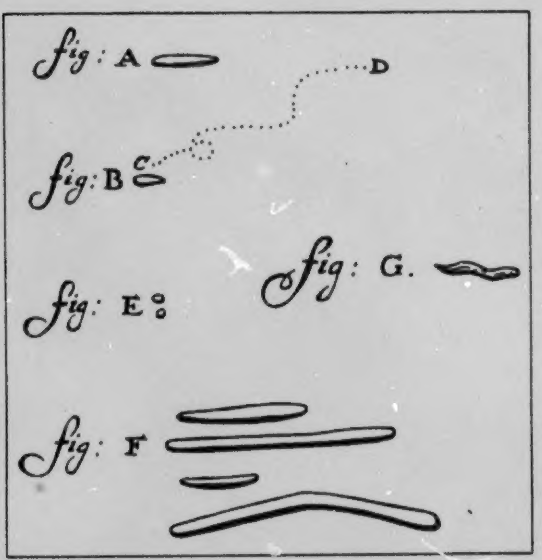


Figure 1. Leeuwenhoek's drawings of bacteria.

Fracastoro published a poem which recounted the legend of the shepherd Syphilus who had been afflicted with the disease. The name is still with us.

1-4. Development of the microscope was probably the most significant occurrence in the field of bacteriology. The first simple lens was made by Roger Bacon. This invention was followed by the compound lens of Zacharias Janssen in Holland. The world of microbes was now visible to all who were interested. The first notable observation of microbes was recorded by Anton van Leeuwenhoek in 1675. With his crude microscope, composed of a biconvex lens, Leeuwenhoek discovered bacteria in water and body fluids, as well as yeast in beer. He saw different shapes and observed that certain bacteria could move from one place to another under their own power. His drawings of these organisms, one of which is shown in figure 1, formed a foundation of the modern-day classification of bacteria. Invention of the microscope provided a means to study the causes of fermentation and disease. Naturally, knowledgeable men of the day wanted to learn more about the identity and functions of microbes. However, they were handicapped in their efforts because Leeuwenhoek jealously guarded the secret of his microscope until his death.

1-5. After the death of Leeuwenhoek, with the aid of this new instrument and with the information of the more than 200 letters which Leeuwenhoek wrote to the Royal Society of London about his experiments and observations, the science of microbiology was started.

2. Contributions of Important Bacteriologists

2-1. Belief in the theory that living organisms could originate from nonliving matter made it easy to explain the presence of biological beings, but hampered research for scientific truths. In fact, *spontaneous generation* was so commonly accepted that one could purchase a recipe book which contained a formula for producing mice by simply placing a pair of old trousers, an old shoe, and a few crumbs of food in a quiet, dark corner! In a few days or so mice would indeed be found in the corner, or at least evidence that they had been there. Fortunately, there were individuals who would not accept such examples as truths.

2-2. **Francesco Redi.** Francesco Redi (1626–1697) wanted to disprove once and for all the theory that putrefying meat gave rise, spontaneously, to living worms or maggots. His simple and classical experiment consisted of three jars into which he placed pieces of fresh meat. He left one jar open, thus exposing the meat to air and flies. Another he covered with gauze; while still another be sealed with a tight-fitting lid. He noticed that flies visited the jars which were exposed to the air, and indeed observed that white worms began forming on the meat in the jar left uncovered. White worms were also found on the gauze which covered one of the jars. No worms were found in the tightly sealed jar although putrefaction was evident.

2-3. Redi's experiment started the beginning of the end for the theory of spontaneous generation. This experiment was scientific proof for the day. The worms or maggots were simply fly larvae, which eventually turned into flies. Of course, advocates of spontaneous generation tried to discredit this evidence, but their efforts were to no avail.

2-4. **Lazzaro Spallanzani.** Lazzaro Spallanzani (1729–1799), interested in the work of Redi and others, approached the problem of spantaneous generation in another way. He boiled and sealed in glass jars various infusion broths made from vegetables and meats. He noticed that no putrefaction or other apparent changes occurred in the broths even after long periods of time. This experiment was discredited by the spontaneous generation advocates who claimed that the heat had destroyed the air. Other scientific minds would later apply Spallanzani's principle to preserve food stuffs, resulting in benefits we still enjoy today.

2-5. **Schroeder and Von Dusch.** To further disprove the theory of spontaneous generation, two scientists, Schroeder and Von Dusch, followed up the experiments of boiled infusions. However, instead of sealing, they closed the containers with gauze plugs, allowing air to enter the vessels. Eventually some decomposition took place in the broths naturally. This led Schroeder to wonder if the broth changes were caused by organized microscopic germs, or some unknown chemical substance. The decomposition was later attributed to oxygen. The cotton plugs had kept bacteria, flies, and other particles out of the broth and are still used to great advantage in modern day bacteriology.

2-6. **Edward Jenner.** The 18th century saw many innovations and scholarly contributions to the science of microbiology. Particularly important were the discoveries of Edward Jenner (1749–1823) regarding immune principles. His classical experiments in developing and administering vaccines are known worldwide.

2-7. **Joseph Lister.** The 19th century saw the use of antiseptics in surgery. The use of antiseptics in surgery was advocated by Lord Joseph Lister (1827–1912). He proposed the theory that "infection was due to passage of minute bodies capable of self-multiplication from infector to infected." He soaked ligatures in disinfectants and even went to the extreme of performing operations under a spray of phenol in an effort to destroy and/or prevent these minute bodies from infecting the patient. Because of his many contributions, the title, "Father of Aseptic Surgery" was bestowed upon him. One of his least-known credits, although questionable, is that of being the first person to obtain a pure culture of a microorganism.

2-8. **Louis Pasteur.** Probably the greatest blow to the theory of spontaneous generation was dealt by Louis Pasteur (1822–1895). Pasteur's simple experiment, using flasks with long, swan-neck, tubular openings revealed that contamination of nutrient material could only take place when the material had access to air laden with living microorganisms. As the air passed through the tubular neck, the lack of air currents prevented the organisms from rising up the tube and gaining access to the nutrient material within the flask. Consequently, the flasks remained uncontaminated.

2-9. Louis Pasteur is well-known for his work on fermentation, rabies, and anthrax. Pasteurization of many of our food stuffs is a result of his experiments and observations. A major contributor to the "Golden Age of Bacteriology," Pasteur is recognized as the father of bacteriology. In disproving the theory of spontaneous generation, he developed procedures which are basic in microbiology today.

2-10. **Robert Koch.** Knowledge of bacteria is important, and bacteriological techniques are of prime concern to clinical bacteriologists. The work of Robert Koch (1843–1910) provided a firm foundation for modern-day techniques. He discovered and developed the use of culture media, was able to isolate pure cultures of microorganisms, and introduce the rapid-air drying of thin bacterial films for staining. In laboratory research and development of bacteriological techniques, he established valid criteria for determining the cause of infectious disease. These criteria are known as "Koch's Postulates" and form the basis for the identification of many bacteria. His classic work with tuberculosis stands as a tribute to these postulates. These postulates, briefly stated, are:

- A specific organism must always be associated with a specific disease.
- The organism must be isolated in a pure culture.
- The organisms must cause the specific disease in a susceptible animal.
- The organism must, in turn, be isolated in pure culture from the diseased animal.

2-11. Improved bacteriological techniques make it unnecessary to go through each step of Koch's postulates in laboratory diagnosis of most disease agents. There are many organisms which do not produce disease in experimental animals. Furthermore, certain organisms in pure culture require close association with another organism in order to produce infection. These postulates have made a science out of microbiology and are mandatory requirements for successful investigation of certain diseases.

2-12. Through careful observation and recording of experimental results, you can make valid conclusions in the bacteriology laboratory. It requires practice, perserverence, and astuteness to become a successful clinical bacteriologist upon whom the physician and patient rely for determining the cause of an infectious disease. You, as a medical laboratory technician must be constantly alert to new discoveries and concepts which improve medical bacteriological techniques. The words of Pasteur (1854) admirably illustrate this point: "Without theory, practice is only routine; governed by the force of habit. Only theory can breed and develop the spirit of invention." Perhaps in this course, we can stimulate and develop the spirit of invention in you.

CHAPTER 2

Aseptic Techniques and Sterilization Procedures

SAFETY OF PERSONNEL is of prime concern in any task we do. It is a major concern in microbiology because of the nature of the material we work with. Every specimen sent to the laboratory is a potential carrier of disease-producing organisms, and as you become more proficient you might become careless. Through your carelessness you can infect yourself with a disease-producing organism, or worse yet, become a walking carrier of a disease and spread the disease to unsuspecting persons.

2. You must know and use proper safety procedures in collecting, handling, and processing specimens to protect yourself and the patient from further disease. You must be able to handle the specimen within the laboratory without causing contamination of your co-workers. Also, you must be able to decontaminate a contaminated area, give first aid in case of accidental contamination of yourself or a co-worker, and take the right steps to report accidents.

3. Specimens are received in the laboratory in a variety of containers and by many different means. You must know the various types of containers in use, their advantages, disadvantages, and methods of sterilizing them before and after use. You must know how to properly dispose of materials after you have finished with them. Also, you must know how to ship a specimen properly to avoid breakage and contamination. As we go along, we will discuss pertinent safety precautions as they apply to individual procedures.

3. Procedures for Receiving and Collecting Specimens

3-1. Before a specimen is processed in your microbiology laboratory, you must either receive it or collect it. Most often, it is sent to you after it has been collected by a physician or nurse. On other occasions, the patient comes to the laboratory and you collect it yourself. Let's look at a specimen that you might receive.

3-2. **Receiving Specimens.** All specimens received in the laboratory must be accompanied by the proper Standard Form (SF 514 series), attached to the container. These forms should be prepared in duplicate by the patient's physician. Most often, they are prepared from the doctor's orders by the ward nurse, the senior medical service technician, or similar personnel in the clinic service. If the physician has signed the DD Form 728, Doctor's Orders, he does not need to personally sign the laboratory request.

3-3. All forms accompanying the specimens should have the appropriate blocks filled in with the pertinent information. Properly completed forms, with attention paid to possible diagnosis and antibiotic therapy, will aid you in isolating pathogenic bacteria with a minimum loss of time and greater efficiency.

3-4. Each specimen container should be labeled in such a way that if the request form is separated from the specimen, the two can be quickly identified as belonging to each other. The laboratory officer will designate the appropriate label for each type of container. The label should be completed by ward or clinic personnel and should show the name of the patient, the register number, the ward location (bed number, etc.), the patient status, and any other significant data. It is possible to have patients on a ward who have the same first names, last names, and middle initials. If the other data are incomplete or incorrect, a mixup can occur, and you will not be able to tell which specimen belongs to which patient. This can be of serious concern to the physician who is trying to make a positive diagnosis, and to the patient he is treating. You, the laboratory technician, should always check the container label to assure yourself that the container and its contents do go with the request form.

3-5. The examination desired should be indicated by a check in the appropriate blocks on the form or by writing in any special requests. When a specimen is received in the laboratory, check the request form to insure that you know exactly what is requested. To aid you, and for future reference, the anatomical site from which a specimen has been taken should be adequately described. For example, you would want to know if it is a specimen of "pus from wound of right thigh," "purulent pleural fluid from the right side of thorax," or "fluid from abdomen." If you know the source, you can decide what steps to take to isolate and identify the disease-causing organism.

3-6. **Rules for Collecting and Processing Specimens.** Collecting a specimen can often be your responsibility. At other times, you may have to give instructions for proper collection. Improper collecting and processing of bacteriology specimens have been definite factors in the failure to isolate and identify the bacterial agent of a disease. The following are some simple rules for collecting and processing bacteriological specimens:

a. Instruments, containers, and other equipment coming in direct contact with most specimens must be *sterile* (the stool specimen is one exception). Material for culture must not come in contact with chemicals, disinfectants, or germicides.

b. Material for culture should be obtained before the patient receives antibiotic or sulfonamide therapy. If he has received some type of therapy, it should be written on the request form.

c. Specimens should be properly labeled and dated.

d. Specimens should be delivered to the microbiology laboratory immediately after collection.

e. Specimens should be inoculated to media immediately after delivery to the microbiology laboratory.

f. Anaerobic culture specimens should be inoculated to thioglycollate liquid medium at the time the specimen is collected, or as soon thereafter as possible.

g. To avoid contamination, culture the specimen before making smears or performing special tests.

3-7. *Blood specimens.* Isolation of microorganisms from blood can help the physician to diagnose and treat various infections. Invasion of the blood may be transitory or fulminating. It depends upon the disease agent, the primary focal point of infection, and the resistance of the host. The terms, *bacteremia* and *septicemia*, are often used to describe these conditions.

3-8. Many blood cultures yield negative results because samples are not collected at the proper stage of the disease. Specimens of blood should be drawn when symptoms indicate circulatory involvement such as chills, fever, or convulsions. Negative results from a single blood culture do not rule out septicemia.

3-9. You can use many methods to culture blood. Regardless of the method that you choose, the blood should be drawn by venipuncture, using strict aseptic technique. You may immediately inoculate blood into broth at the bedside of the patient, or collect it and take it to the laboratory in sterile, capped test tubes containing sterile anticoagulant.

3-10. *Body fluid specimens.* Body fluids include spinal, synovial, pleural, pericardial, and peritoneal fluid. Examination of spinal fluid is important in determining bacterial meningitis. In many cases, emergency chemotherapeutic agents are administered on the basis of results from a microscopic examination (gram-stained smear) of centrifuged spinal fluid. The genera of microorganisms most frequently responsible for meningitis are *Neisseria, Diplococcus, Streptococcus, Staphylococcus, Hemophilus,* and occasionally *Mycobacterium.* One of the fungi chiefly responsible for a type of meningitis is *Cryptococcus neoformans (Torula). Neisseria* may be found in specimens of synovial fluid from arthritic patients.

3-11. When you receive a body fluid for culture, you should enter the source, provisional diagnosis, as well as any antibiotic therapy on the request form SF 514K, Bacteriology. This will help you to select the proper media for inoculation. All body fluids are aspirated by a physician and transported to the laboratory in sterile, screw-capped test tubes. You should not waste any time between collecting and culturing body fluids. These fluids contain enough fibrinogen to coagulate the fluid if we delay. Always practice extreme care when handling body fluids, for these fluids often contain highly infectious organisms.

3-12. *Exudate specimens.* An exudate is material that has passed through the walls of vessels into nearby tissues or areas of inflammation. Exudates may come from boils, wounds, ear or mastoid infections, eye infections, and skin lesions.

3-13. A great variety of microorganisms may be isolated from such areas. Boils may yield *Staphylococcus* spp., *Streptococcus* spp., and occasionally Gram-negative bacilli such as *Escherichia* spp., *Pseudomonas* spp., or *Proteus* spp. Deep wounds, especially puncture wounds, provide ideal living conditions for anaerobes, especially *Clostridium* spp. An infected wound that exhibits a greenish or bluish purulent discharge may be a *Pseudomonas* infection. Exudates from surgical or postoperative infections may reveal anaerobic *Streptococcus* spp. Ear and mastoid infections are often caused by *Pseudomonas*, *Staphylococcus*, or *Streptococcus* spp. Eye infections may yield *Hemophilus* or possibly *Neisseria* spp. Purulent discharges from the urethra frequently reveal *Neisseria gonorrheae*. A Gram-stained smear of exudate from a chanchroid or soft chancre, usually reveals small Gram-negative rods of *Hemophilus ducreyi*.

3-14. You may collect exudate material in the form of purulent drippings from cases of urethritis in male patients on sterile, cotton-tipped swabs or on sterile wire bacteriological loops and inoculated directly into culture media. If you collect the urethral discharge for examination for gonorrhea, use the utmost discretion and tact and give the patient privacy. Collect the specimen with the least amount of embarrassment and talk. When you see the request, do not broadcast it for all to hear. Remember always that laboratory requests and results are privileged, confidential information and this information is between you and the physician only.

3-15. *Fecal specimens*. Bacteriological examination of *fecal specimens* aids diagnosis of gastrointestinal infections manifested by diarrhea and/or dysentery. Stool cultures, along with blood and urine cultures, are important aids in diagnosing typhoid and paratyphoid fevers. Many diseases are spread by human carriers through food and drink. Therefore, we perform stool cultures on food-handling personnel to supplement public health control measures.

3-16. The organisms most frequently involved in enteric infections are the *Salmonella* and *Shigella* spp. *Arizona arizonii* has been implicated to a lesser degree. The normal intestinal flora of the adult are primarily *Escherichia coli (E. coli)*, *Citrobacter* spp., *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Bethesda-Ballerup* and *Providence* groups. *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, and *Proteus* spp. are sporadically present and may be called transient saprophytes. Saprophytic organisms, including *Proteus morganii* and the *Providence* group, have been implicated as etiological agents of infant diarrhea, as has pathogenic *E. coli*.

3-17. In culturing specimens of intestinal origin, the basic problem is to isolate pathogenic agents from specimens which may contain large numbers of saprophytic organisms. To do this, we use special, differential, selective, and inhibitory media.

3-18. Fecal specimens should be collected in clean, wide-mouth containers with tight-fitting lids. Cardboard half-pint containers with tight-fitting lids are generally used. The advantage of their use is that the containers and contents can be incinerated. Stool collection containers do not have to be sterile, but they should always be clean. You should never mix specimens with body fluids, or collect them from bedpans or commodes.

3-19. If you use rectal swabs for a specimen, they should be sent to the microbiology laboratory in clean, cotton-plugged test tubes. Rectal swabs are useful to collect cultures from infants or large numbers of patients. You should culture fecal material as soon as possible after collection. If culturing is delayed, you may not isolate causative agents, particularly *Shigella* spp., as they die off rapidly after collection.

3-20. *Sputum specimens*. Sputum specimens are usually from true or suspected cases of pneumonia or tuberculosis. You will use different techniques to examine sputum specimens for bacteria that cause penumonia and bacteria that cause tuberculosis. You must be sure that the request clearly states the type of examination to be performed. If in doubt, check with the physician.

3-21. You should collect sputum for bacteriological examination in sterile, wide-mouth, screw-capped jars. In cases of pneumonia, one sputum specimen is usually enough for the examination. Collect the specimen as soon as the patient awakes in the morning. You should explain to the patient the difference between sputum and spittle. Sputum is a secretion brought up from the lungs and bronchial tree, and is usually associated with a cough. Spittle is nothing more than an accumulation of saliva in the mouth. Spittle will not show the bacteria infecting the deeper regions of the respiratory system.

3-22. *Throat and nasopharyngeal specimens*. Throat and nasopharyngeal cultures help diagnose infections such as streptococcal sore throat, scarlet fever, diphtheria, and whooping cough. They can determine the focal point of infection in diseases such as rheumatic fever and acute glomerulonephritis. In epidemiological studies, these cultures can detect carriers of beta hemolytic *Streptococcus*, hospital *Staphylococcus* spp., *Corynebacterium diphtheriae*, and other potential pathogens.

3-23. Collect throat and nasopharyngeal specimens under good lighting with sterile, cotton-tipped swabs as shown in figure 2. You can also use a flexible wire, cotton-tipped West swab as shown in figure 3. The culture tube in which the swab is placed may contain sterile culture broth to prevent drying of the specimen. Thioglycollate, trypticase soy, or Todd-Hewitt broth is satisfactory for this purpose. In figure 4, we show how

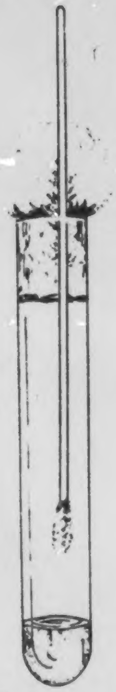


Figure 2. Throat culture collection tube with swab.

to depress the tongue with a tongue blade and pass the swab gently past the crypts and tonsils (4A) across to the back surface of the pharynx (4B). Do not touch the swab to the tongue, cheek, or teeth. The tongue, cheek, and teeth harbor a variety of organisms unrelated to the disease organisms which may be pathogenic and significant if isolated from the proper site. A swab contaminated by contact with the tongue, cheek, or teeth may add many confusing organisms and make isolation of the pathogen difficult. Place the swab in a sterile test tube and immediately send the specimen to the bacteriology laboratory.

3-24. When you receive a cotton-tipped swab for culture and smear, you must remember to make cultures before making the smear. Otherwise, bacteria may be transferred from the smear slide to the culture media and we have contaminated the culture. You should discard the swabs into a solution of disinfectant. The addition of swabs to the solution tends to dilute it, thereby, lessening its effectiveness, and some spores are not destroyed. Therefore, you should sterilize the swabs and container in the autoclave and *not* discarded directly into a trash can. Remember, all material received for bacteriological examination is a potential hazard and can infect you and all others who may contact the material. Use caution when disposing of such material.

3-25. *Urine specimens.* Urine cultures are of value in diagnosing primary infections of the urethera (urethritis), bladder (cystitis), and kidneys (nephritis). They are also important in diagnosing certain systemic infections caused by bacteria excreted via the urinary tract. *Staphylococcus* spp., *Streptococcus* spp., and *Neisseria gonorrheae* are the primary etiological agents of urethritis; while *Escherichia, Proteus,* and occasionally *Pseudomonas* spp. are causative agents of cystitis. Any one or more of these organisms may cause a bacterial nephritis. Yet, many of these organisms may be common urine contaminants. A physician usually does not make a diagnosis on the basis of one urine culture. Rather, repeated isolation of large numbers of a particular organism from a series of urine specimens is evidence for the pathogenicity of an organism.

3-26. Ideally, catheterized morning specimens obtained aseptically are desired for all routine bacterial examinations of urine. Voided specimens are likely to contain contaminating saprophytes. Where male patients are concerned, following proper cleansing of the glans penis, it is permissible to collect a clean (midstream), voided urine specimen. Urine from female patients is best obtained by catheterization since voided urine may be contaminated with bacterial

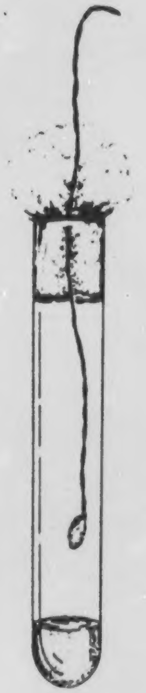


Figure 3. West swab for collecting nasopharyngeal specimens.

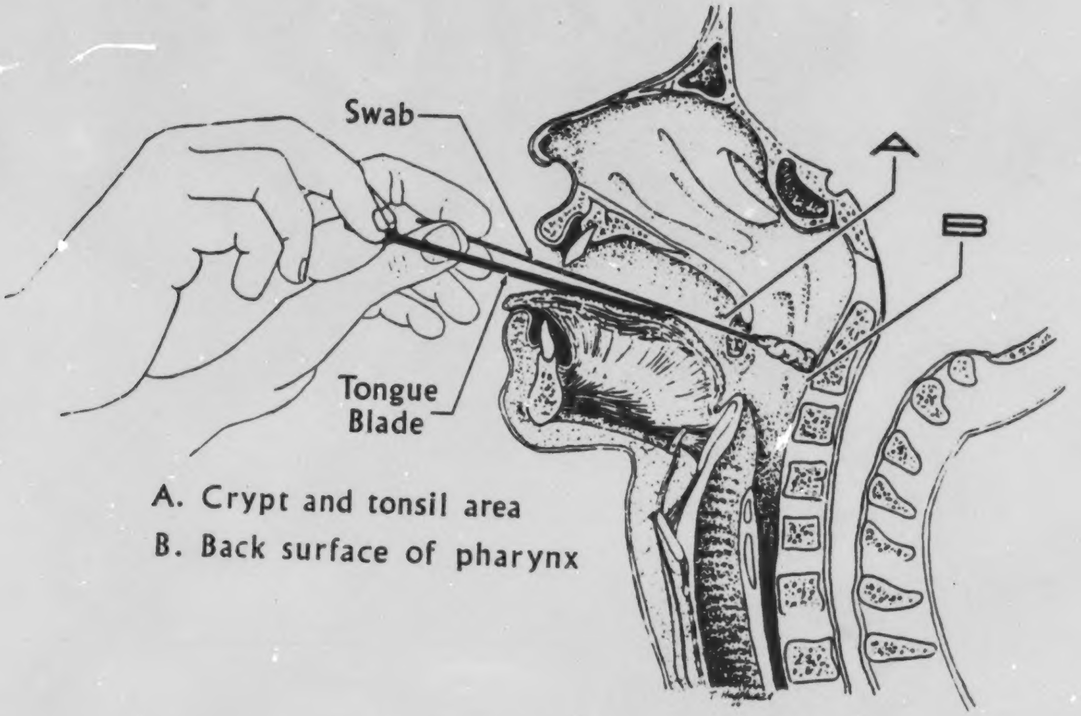


Figure 4. Collection of throat swab cultures.

flora present in the external genitalia or from vaginal discharges. You should collect all specimens in sterile, urine bottles or sterile, widemouth, screw-capped jars. Delay between the collecting and culturing of urine specimens may contribute to a change in pH and other characteristics of the specimen, resulting in a high death rate of the organisms present.

3-27. Urine, unless catheterized, should be collected by the patient himself. This may be done on the ward or the patient may be sent to the laboratory with the proper request form. The physician will state whether he wants a clean-voided, midstream, two-glass, or three-glass urine specimen depending upon what specific information he is seeking. You must have knowledge of these terms and know what is meant by them if the proper collection is to be made.

a. To collect a *clean-voided* urine specimen, the patient is instructed how to wash the genitalia with a suitable cleansing solution. He should void directly into a sterile, screw-capped container without contaminating the inside of the container.

b. When the physician orders a *midstream* urine specimen, the patient is instructed how to wash the genitalia with a suitable cleansing solution and to void—discarding the first part of the urine stream. He is told to collect, in a sterile container, that part of the urine stream which, in his opinion, is the middle third. The last part of the urine is also voided and discarded. The patient must understand that it is the middle third of the urine stream that is desired.

c. Sometimes the physician will order a *two-glass* or *three-glass* urine specimen. When either of these is ordered, the patient is instructed how to wash the genitalia with a suitable cleansing solution and to collect the urine in two or three sterile, screw-capped containers. The patient is given numbered bottles and told to collect the specimens in the order the bottles are numbered. The volume in each bottle does not matter; no attempt should be made to adjust the volumes by pouring urine from one bottle to another. The volumes should be left as collected.

3-28. To avoid embarrassment to yourself and the patient, it is best to have a printed set of instructions for each of the collections. When the patient hands you a request form, all you need to do is see that the patient has access to containers, cleansing material, and a private place to collect the specimens. If the patient is a child, give the instructions to the accompanying adult, who in turn can see to it that the specimen is properly collected.

20

3-29. There are valid reasons for collecting the urine specimen as described above. A clean-voided urine can indicate an infection in the urethra or the anterior section of the bladder. The midstream collection helps to pinpoint an infection in the bladder. The three-glass urine divides the urine as coming from three separate anatomical sections of the urinary system. The first is the urethra, the second is the bladder, and the third is the anterior part of the bladder, or possibly the urethers and kidney, as some urine may drain into the bladder when it empties.

3-30. Other methods of urine collection are done by the physician. These methods include catheterization of the bladder, the ureters (such urine comes to the laboratory in marked containers, *right ureter* and *left ureter*), and possibly from the kidney itself. You must remember to handle each specimen carefully and to avoid mislabeling your cultures.

4. Handling Microbiology Specimens

4-1. All specimens received in the laboratory should be in containers which have a clean, uncontaminated exterior surface. It is possible that during delivery of the specimen from the ward, place of collection, or within the laboratory itself, splashing of the contents may occur. If the container does not have a tight-fitting lid, some of the contents may seep around the cover and contaminate the outer surface. This contaminated surface is a source of infection to you or anyone else in the laboratory. Do not accept any specimen which shows evidence of a contaminated outer surface.

4-2. Wrapped specimens (a common practice with outpatients), should be unwrapped and removed from their wrappers by the person bringing the specimens to the laboratory. Do not let the person leave until you are sure that a request slip has accompanied the specimens and that the specimens are properly identified.

4-3. A diaper is sometimes brought to the laboratory for bacteriological examination of its contents. Check the request form to see if the physician is interested in enteric bacteria, then the diaper contents are acceptable if the specimen is fairly fresh. If the physician wants a check for bacteria other than enteric organisms, another fresh specimen should be collected in a sterile container or on a rectal swab. Do not rinse the diaper, as this subjects you to a source of contamination.

4-4. As an added safety precaution, it is wise to use a bacteria isolation hood wher. working with highly contagious material. You should also wear a face mask and rubber gloves to prevent contaminating yourself. Perform all bacteriological work on a nonabsorbent surface. You should place a disinfectant-soaked paper towel or similar absorbing material directly on your working surface. This will minimize contamination caused by droplets or spillage of a specimen. When you are finished with your work, the entire area should be wiped down with a disinfectant.

4-5. When you mix liquid specimens or serially dilute broth cultures, BE VERY CAREFUL. The aerosol produced during these procedures is a source of danger and can very easily spread a "fog" of bacteria throughout the immediate area. The aerosol can infect you or another person through the natural process of breathing. The bacteria may not settle until after you have cleaned up and left the area.

4-6. Occasionally, you may centrifuge a specimen or culture to concentrate the material. To do this, you use tightly fitted screw-capped tubes to prevent contamination of the material as well as the atmosphere. However, you must be careful in the choice and care of glassware. Check it before use for cracks and chips. Make sure one tube is balanced with another tube before spinning. Also, it is important to check your centrifuge for balance. Unbalanced, the tubes may get broken, spreading dangerous biological material all over the laboratory, or centrifugal force may tear the head from the motor shaft. Do not exceed the recommended speeds set by the manufacturer of the centrifuge or the glass maker.

4-7. You should stuff nonabsorbent cotton into the mouthpiece of all pipettes used in bacteriology. The cotton should not fit so tight that a great amount of suction is needed to draw the fluid into the pipette. You might suck the cotton out of the pipette and get a mouthful of bacteria for your efforts. A commercial rubber suction bulb which is easily controlled with the hand should be used with pipettes. Here again, you must exercise care and not contaminate the bulb.

4-8. Remember, the steps you take to prevent contamination of yourself, other persons, and the laboratory are worth the effort. It takes less time to prevent contamination than it does to treat a person who becomes infected.

4-9. **Types of Containers.** There are many factors to consider when choosing a container for a bacteriology specimen. The type of specimen, source of specimen, analysis desired, time lapse between collection and media inoculation, and the final disposition of the container are important factors. Each laboratory, including the one in which you are now working, has an established procedure for selecting containers used to collect the various specimens. Learn and use them only as directed. Remember to consider the above points in choosing containers for specimens.

4-10. The *type of specimen* will be our first dictate in choosing a container. Naturally, the viscosity of a specimen will play a part in our choice between a cardboard or a glass container, and between a loose-fitting or tight-fitting lid. You should collect fluid specimens such as urine, spinal fluid, and pleural fluid in a screw-cap, sterile, glass container. Glass is easily sterilized, and is, therefore, an excellent container for most specimens. Wax-coated cardboard containers cannot be sterilized with heat and, therefore, must be used sparingly. However, they are well suited to collect stool specimens. The box and its contents can be readily incinerated after use.

4-11. Certain body fluids may coagulate before they get to the laboratory. In this case, you would use a sterile, screw-capped, wide-mouth glass jar. You would culture the whole clot, because as the clot forms, bacteria along with cellular structures such as white blood cells, red blood cells, and epithelial cells, will be caught in the fibrin clot. If you want to inhibit clotting, you can use anticoagulants. They must be sterile, compatible with bacterial life, and used according to the manufacturer's directions. You can avoid clotting by inoculating the culture media directly at bedside as the specimen is collected.

4-12. A needle with a large eye is easier to thread than a needle with a small one. Likewise, a wide-mouth container to collect a sample is easier to use than a narrow-mouth container. And so, we must consider the *source of the specimen*. Urine, sputum, tissues, exudates, or specimens of large quantity should be collected in various sizes of screw-capped, wide-mouth containers. Spinal fluid, small quantities of urine, and cavity fluids can be collected in small, screw-capped tubes.

4-13. If you catheterize to obtain the urine, use a sterile, screw-capped test tube. Test tubes are easy to centrifuge, so the specimen need not be transferred to a tube for centrifugation, thereby, eliminating a step which could introduce a source of contamination. You should also collect spinal fluid, because of its usual small volume, in sterile, screw-capped test tubes.

4-14. The *type of analysis* requested should be considered before choosing the type of container. Again, we refer to stool examination. Normally, we use a clean, waxed cardboard box. However, you must use a sterile container to collect stools if you are interested in other than enteric organisms. To study various body fluids for virus and rickettsial-like organisms, may require that you collect two or more specimens a few weeks apart. This means storing one specimen until you receive the second one.

4-15. If a suitably sized container is not selected, we may quickly run out of storage space for sterile and nonsterile containers. Occasionally, 24-hour urine specimens are collected to check for tuberculosis of the kidneys. Storage of containers large enough for this volume can pose a problem to both the ward and the laboratory. Selection of suitable containers is based on intelligent forethought as to storage space.

4-16. Another important factor is the *time lapse* between getting the specimen and culturing it. It is desirable to transfer the clinical material to a suitable growth environment as soon as possible after collection, but it is possible to hold some material for a relatively long period of time. As an example, outpatient clinics collect throat and nasopharyngeal culture swabs. Instead of these swabs going to the laboratory as soon as collected, they may be kept at the clinic for several hours. Let's face it! It is better to make 1 trip carrying 10 or 12 tubes to the laboratory than it is to make 10 or 12 trips carrying 1 tube each trip.

4-17. If too much time elapses between collection and delivery, the swabs may dry out and the chances of recovering pathogenic organisms are greatly reduced. To prevent this happening, you should place the swabs in a tube of broth or other "holding" material. In addition to preventing drying, there is the advantage of giving the bacteria a headstart in their growth. In turn, this means that the physician gets his report back quicker and the patient is assured of faster treatment.

4-18. Do not allow a specimen to stand a great length of time before starting the necessary studies. Some fluids, if allowed to stand, will change in their state of acidity or alkalinity. This change may cause some bacteria to die while other bacteria grow and multiply faster. This means that a pathogenic organism with a slow growth rate may be overgrown by a fast-growing nonpathogen. Current research is investigating the possibility of allowing cotton swabs to dry in order to eliminate saprophytes. As this technique is not part of this section, we will not discuss this work here. It is important to start the identification procedures as soon as possible. We must impress our ward and clinic personnel with the idea that the specimens must get to the laboratory with the least amount of delay.

4-19. After all is said and done, we have to dispose of the biological material. By *final disposition* we mean—"What will happen to the container and the contents when we are finished with it?" Do not discard any contagious material into a common trash can. Containers such as plastic tubes, plastic bottles, and wax-coated cardboard containers can and should be incinerated. You should sterilize glass containers by autoclaving before the container and its contents are discarded. Your laboratory has a standard procedure for discarding contaminated containers and material. Know and follow it to the letter!

4-20. The advantages and disadvantages of most containers center around ease of sterilization. Glass containers can be easily sterilized by autoclaving, by hot-air oven, or by the simple expediency of boiling them in water for the proper length of time. This latter technique means that outpatients can sterilize their own bottles at home if necessary.

4-21. Plastic and cardboard containers are not easily heat-sterilized. They can be sterilized with certain gases, such as ethylene oxide, but most laboratories do not have access to this type sterilizer. The use of disposable containers is encouraged for bacteriological work. But again, there is a logistic problem involved with their use and often we cannot keep enough of the necessary supplies on hand. It is essential that you know the container types, their uses, and methods of sterilization in order to choose a container style which best meets the needs of your laboratory.

4-22. **Preservation.** As a bacteriology technician, be certain that you have performed all the procedures the physician requested. Be absolutely certain that you start all procedures as soon as the specimen is received in the laboratory. Only after you are assured that you have done all that is required, may the specimen be discarded. However, you may find it necessary to preserve the specimen for future reference. Preserving bacterial specimens must be done properly to insure correct identification of the organisms at some future date. There are several methods of preservation; the one you use will depend on why you are preserving the specimen. We will discuss the methods of freezing, freeze-drying, refrigeration, dessicating, chemicals, and the use of holding media.

4-23. *Freezing.* You can freeze bacterial specimens for a long time at temperatures of −70° C. or colder. You must be aware of the factors which favor survival or cause death of microorganisms subjected to low temperatures. You should freeze bacteria very carefully. As bacteria freeze, ice crystals or spicules form inside the cell. Large crystals pierce the cell wall and cause mechanical destruction of the organism. If you freeze the organism rapidly, you can prevent the large intracellular crystals and favor the formation of small crystals. Some authorities believe that slow-freezing is preferred because time is allowed for the osmotic pressure adjustments between the interior of the cell and the surrounding fluid. Freezing can be done in a solution of 5 to 50 percent glycerol in saline, or in any other solution which will slowly remove the water from the cell and prevent crystallization.

4-24. You can use the freezer section of the refrigerator to freeze specimens for viral examination, whereas, you usually cannot use it to freeze and preserve bacteria. For viral studies, you should collect at least two specimens and examine them simultaneously. The first specimen is marked "acute sample," and is collected while the patient has the suspected disease. The second specimen is marked "convalescent sample," and is collected 2 to 3 weeks later, after the disease has subsided and antibodies have had a chance to build up within the body. Process both samples together. As a diagnostic tool, neither sample is good without the other. The first specimen is preserved by freezing; first, to prevent overgrowth by bacterial contaminants, and second, to slow the metabolism of the virus. Store the acute specimen in the freezer compartment of the refrigerator, pending receipt of the convalescent specimen. Since the stability of any viral agent is unknown, freezing is considered only a temporary measure. We will cover a more complete discussion on specimens for viral studies in a later volume.

4-25. *Freeze drying.* The method of freeze-drying depends upon extreme cold, rapid evaporation of water, and the use of a vacuum to eliminate any water which may crystallize. First, mix the bacteria in a suitable fluid medium and dispense the bacteria in small aliquots to glass ampules. Put the ampules into a bath of carbon dioxide ice and alcohol or acetone to reduce the temperature to near −76° C. The contents will freeze immediately. The contents are dried while they are in the frozen state by using a high vacuum which will sublimate the water. The ampules are heat sealed while still under vacuum. To reconstitute this powder, you need only add a sterile broth medium or other sterile fluid.

4-26. The preservation of bacteria by using temperatures ranging from 0° C. to −76° C. is probably most important to the research or teaching laboratory. Many bacteria and mold forms have been kept for years at these low temperatures. In fact, some species of bacteria will grow even after being subjected to temperatures as low as −252° C. Future examination for reference purposes is then possible.

4-27. *Refrigeration.* Refrigeration at 4° C. will slow the metabolic processes of bacteria to the point where multiplication is negligible. By this means the number, size, and spacing of bacterial colonies can be temporarily preserved. If you must store a culture for a day or so because of lack of media, a holiday, or other reason, you can preserve it by refrigeration.

4-28. *Desiccation.* This method means removing water by drying, and can be used to preserve certain bacteria. Once preserved in this manner, they can survive for long periods of time. It must be recognized that some species of bacteria, however, are readily killed by desiccating procedures and the choice of this method of preservation must be carefully made. Desiccated cultures are convenient because a large number of them may be stored in a small area. Maintained in this manner, those bacteria which survive drying do not produce mutations upon being reconstituted. The simple addition of a sterile media will reconstitute the bacteria. *Corynebacterium diphtheriae* have survived as long as 15 years, *Mycobacterium tuberclosis* as long as 17 years, and pathogenic beta *Streptococci* for 25 years.

4-29. *Chemicals.* Straight chemical preservatives should not be used to preserve bacteria. A chemical preservative can alter the bacteria metabolism and in many instances cause death. Consequently, the chances of isolating bacteria from these specimens are not good. If you use a container which has held a chemical preservative, you must clean and sterilize it before using it to collect specimens for microbiological examination.

4-30. You may use glycerine (15 to 20 percent in sterile, buffered saline) to preserve well-formed specimens, such as stools and tissue, but is of little value for preserving liquid specimens. We mentioned in a previous paragraph that 5 to 50 percent glycerine in saline can be used to freeze bacteria.

4-31. *Holding media.* A holding or transport medium should be used to preserve the viability or maintain the original number of bacteria in material of human origin. A medium such as that devised by Stuart works well. A good holding medium should contain a non-nutrient, soft agar to prevent overgrowth; charcoal to neutralize certain bacterial inhibitors; and a reducing agent to prevent oxidation of the medium components. A holding medium is not necessarily a long-term means of preserving bacteria. Eventually, most bacteria die due to a change in the medium caused by moisture loss. Holding medium lends itself well for shipping bacteria to reference laboratories for confirmation and keeping reference cultures in your own laboratory.

4-32. **Shipment.** Many laboratories ship bacterial specimens to reference laboratories for initial analysis or for confirmation studies. All specimens shipped must be accompanied by the properly completed SF 514K, Bacteriology.

4-33. Shipping specimens requires a knowledge of the specimen itself, U.S. Air Force directives, and United States postal regulations. We will not cover this material nor duplicate material which is readily available. Your laboratory should have AFM 160–28, *Methods of Preparing Pathologic Specimens for Storage and Shipment.* As a student and future NCOIC, you should obtain a copy of this manual and become aware of its contents. It is your guide for the storage and shipment of specimens. The manual does not give specific instructions for collecting and processing bacteriological specimens for shipment. We present an outline for processing various bacteriological specimens in table 1. It is also your responsibility to know the specific requirements of the reference laboratories you use. Write or call them for their operating instructions concerning shipment of specimens.

4-34. **Precautions Against Contamination and First Aid.** In any highly technical field, you need to be very careful and know the hazards that exist. In microbiology, you need to use more than ordinary care to avoid trouble. You can become infected or infect others with sloppy techniques or through carelessness.

4-35. *Precaution against contamination.* At times your work areas will become contaminated with pathogenic organisms. This occurs as a result of human carelessness and accidental breakage of containers due to flaws or aerosol sprays. To prevent the spread of a disease agent from the contaminated area, it is essential that you start decontamination procedures immediately. Knowledge of these procedures is a necessary part of your job.

4-36. You can best carry out surface decontamination by using a phenol solution. Phenol compounds are absorbed as a thin, fairly durable film on surfaces to which they are applied. This film creates a residual effect and besides destroying bacteria already present on the surface, serves to destroy bacteria which land on the surface for several hours after it is applied.

4-37. Halogens, heavy metals, synthetic detergents, and alcohols work well for the first decontamination, but as a rule, lack the ability to remain potent for any period of time.

4-38. If you fail to decontaminate an area immediately or as soon as possible after contamination, this may result in contaminating a much larger area, yourself, your work, and everyone in the vicinity because of air currents caused by movement of equipment and personnel within the laboratory. If you think that an area has become contaminated, decontaminate immediately. Do not wait until you have finished with your work: it may then be too late.

4-39. *First aid procedures.* First aid procedures in a bacteriology laboratory should be limited to those procedures which will prevent infection and preserve life before you can see a physician. **You must know the methods of artificial respiration, the pressure points, how to stop hemorrhage, and how to treat shock. Attend any and all cross-training courses to increase your knowledge and proficiency in these areas.**

4-40. Most accidents are not serious and include lacerations, needle punctures, spillage of contaminated materials, and the improper use of

TABLE 1

COLLECTION AND PROCESSING OF BACTERIOLOGICAL SPECIMENS FOR SHIPMENT

Specimen	*Processing for Shipment*	*Remarks*
Ascitic fluid for bacterial and fungal culture.	Place aseptically in sterile containers without preservative.	Where tuberculosis is suspected, see directions for tuberculosis specimens.
Bacteria, pure cultures of, for identification, confirmation and sensitivity tests	Grow in pure culture on beef infusion agar in screw-cap tubes. Prior to shipment, tighten screw-caps and secure with adhesive tape or by dipping in melted paraffin.	Plant subcultures on agar slants and incubate 12 to 18 hours. A duplicate of each culture should be retained in the laboratory of origin until a final report is rendered.
Blood or urine for culture	Collect 3 ml. of blood aseptically before serum, chemo- or antibiotic therapy has begun and transfer to rubber-stoppered bottle containing hemoglobin-tryptosephospate broth or other suitable broth.	Prior to shipment, incubate specimens for 12 to 24 hours.
Serum for bacterial and cold agglutinins, antibiotic levels, and complement-fixation tests.	Collect blood aseptically in sterile evacuated tubes, filling to capacity.	Allow firm clot to form before packing for shipment. Blood for cold agglutination tests must not be refrigerated until cells are separated from serum.
Cerebrospinal fluid for bacterial culture, and antibiotic and sulfonamide level.	A minimum of 3 ml. of fluid is collected aseptically and inoculated into hemoglobin-tryptosephosphate broth.	Where tuberculosis is suspected, see directions for processing such specimens. Retain broth culture under incubation 12 to 24 hours prior to shipment.
Throat cultures for *C. diphtheriae*	Inoculate Loeffler's serum slant with a fresh throat swab.	Mail specimen immediately.
Exudates for bacterial and fungal cultures.	Place swabs aseptically in sterile screw-capped containers without preservative.	
Fecal specimens for bacterial culture (except tuberculosis).	Emulsify a 5-g. sample aseptically in a sterile screw-capped vessel containing one part C.P. glycerin and three parts 0.6 saline solution. If shigellosis is suspected, immediately freeze the specimen in a solution of alcohol and dry ice. Pack the frozen specimen in cardboard carton with dry ice for shipment.	In food poisoning outbreaks, feces should be collected from patients with diarrhea and all food handlers associated with the outbreak.

TABLE 1 (CONT'D)

COLLECTION AND PROCESSING OF BACTERIOLOGICAL SPECIMENS FOR SHIPMENT (CONT)

Specimen	*Processing for Shipment*	*Remarks*
Specimens for bacteriological examination following food poisoning outbreaks.	(1) Any suspected canned foods should be shipped unopened in original container. (2) Well mixed representative samples of suspected prepared food should be placed aseptically in suitable containers without preservative. (3) Vomitus from all cases in which vomiting is present should be placed aseptically in suitable sized sterile containers without preservative. (4) Swabs from lesions taken from food handlers should be placed in nutrient broth using aseptic technique and transferred to sterile screw-capped test tubes.	Complete epidemiological history should accompany any specimens taken from food poisoning outbreaks.
Joint or other body fluids for bacterial and fungal cultures.	Collect joint and other body fluids and transfer aseptically to suitable sized sterile container without preservative. Plant fluids for mycological culture on Sabouraud's media, or place in sterile screw-capped containers without preservative.	
Sputum for pneumococcus culture or typing.	Collect aseptically before serum, chemo -- or antibiotic therapy is started and place in sterile container without preservative.	
Swabs for culture from open lesions.	Swab lesion with broth-soaked swab and place aseptically in sterile screw-capped test tubes.	
Tuberculosis, specimens for smear, culture, and animal inoculation (other than sputum and gastric contents).	Collect fluids, tissue, or feces in suitable sized sterile containers without preservative.	Specimens include ascitic fluid, cerebrospinal fluid, joint fluid, pleural fluid, pus, urine, lymph node, and other tissue and feces.
Tuberculosis, sputum specimens	Collect all sputum coughed up the morning after first having patient wash teeth and rinse mouth thoroughly with boiled water. Place entire specimen in a sterile wide-mouthed screw-capped bottle; add an equal volume of 20% trisodium phosphate.	During collection, keep jars out of direct sunlight or the tubercle bacillus will be rendered nonviable and, therefore, unsuitable for culture or animal inoculation. *Do not use cardboard sputum cups for collection.*
Tuberculosis, gastric contents.	Through sterile stomach lavage tube introduces 200 to 300 ml. of sterile saline solution into fasting stomach. Evacuate stomach and place entire specimen in suitable sterile container.	Prior to placing specimen in shipping container, neutralize it to litmus with 20 percent sodium bicarbonate.

24

pipettes. In all instances, you need to take first aid steps immediately. After you have taken these steps, refer the individual to a physician and complete an accident report. Ground safety regulations will help you in the proper procedure. Be sure you report all accidents to your NCOIC or OIC.

4-41. Lacerations of the fingers and hands occur frequently, particularly when handling chipped or cracked glassware. If you do cut yourself, you will usually have some bleeding. This flow of blood flushes any bacterial contamination out of the wound. However, you can still become infected because some pathogenic bacteria may enter the bloodstream, where conditions are quite favorable for optimum growth. First aid for a laceration injury is to immediately cleanse the wound with running water, then wrap the wound with sterile material. Proceed to the treatment room to see a physician. It would help the physician to know what organism was on the glassware causing the laceration, or the source of the specimen with which you were working. The physician will be able to better treat the wound and prescribe medications.

4-42. While working with a needle and syringe, it is very easy to stick yourself with the contaminated needle. A puncture wound is a greater danger than a laceration because little or no bleeding occurs with a puncture wound. Pathogenic organisms are forced deep into the tissues. This deeper penetration gives the bacteria an environment where good growth can occur. Don't disregard the fact that you received a needle puncture. See a physician at once, before the signs and symptoms of a disease show themselves.

4-43. Careless spilling of a contaminated fluid can very easily infect you with pathogenic organisms. Although a laceration or puncture does not usually accompany spillage, there is a good chance of contacting a local skin infection because of large numbers of bacteria in a fluid medium, particularly if the material has been incubating for some time. You may have an old laceration and the bacteria can penetrate the healing tissue.

4-44. First aid for spillage is to immediately wash the affected area with a disinfectant-type detergent soap, then rinse with large amounts of running water. You should wash and rinse as soon as possible after spillage, and before you clean up the bench or floor area. Clean and decontaminate the spillage area after you have rinsed the contaminated material off your person. Again, seek the advice of a physician.

4-45. Pipetting accidents are not common in the bacteriology laboratory, but they do occur. There are two major ways in which you might contaminate yourself while using pipettes during bacteriological analyses: (1) misuse of suction and (2) contaminated pipettes. It is very easy to receive a mouthful of contaminated material if you do not watch the level of material in the container while applying suction. If you pull the tip of the pipette out of the fluid, the suction you are applying will draw the fluid in the pipette up through the mouthpiece and into your mouth. This happens quickly and normal reflex action is not enough to prevent contamination. A nonabsorbent cotton plug in the mouthpiece of the pipette can prevent some of this contamination. A rubber bulb to apply suction is safer and is recommended. If you receive a mouthful of contaminated material, rinse your mouth immediately with water and then use a good disinfectant mouthwash. Seek the advice of a physician.

4-46. Another means of contaminating an area is to lay a contaminated pipette down on the bench top. When you finish using a pipette, immediately put it in a disinfectant solution. Do not lay it down on the bench. You might pick up the dirty pipette to use again. This may contaminate your fingers, and when you use your finger or thumb to control the flow of fluid by applying pressure to the mouthpiece, you transfer bacteria to the mouthpiece. Then, sticking the mouthpiece in your mouth will contaminate you. This type of contamination is the hardest to realize because there is no gross reminder of the contamination. Get into the habit of always discarding a used pipette directly into a solution of disinfectant and use a new pipette for each step. *Do not lay a pipette down and pick it up to use again.*

4-47. Contamination of the hands is another source of infection. For this reason, you should always wash your hands before leaving the area. This is a time-consuming procedure, but it is a necessary one if you wish to eliminate a source of danger to everyone you may contact. Smoking is another source of infection. Many technicians will lay their cigarette, cigar, or pipe on the edge of a bench instead of in an ash tray. The subsequent insertion into the mouth can be a source of contamination. Do not smoke, eat, or drink in the procedures area of the laboratory.

4-48. Every laboratory has, or should have, in existence a standard procedure to follow in the event it becomes necessary to report an accident. If you become ill after contamination due to an accident and the illness is attributed to the same organism with which you were previously exposed, diagnosis, treatment, and prognosis of the infection is made easier. This also makes the analysis of your health records for future disabil-

33

ity claims easier to interpret and evaluate. Accident reporting protects you, so don't neglect it. Do not let your co-workers forget it, either. You are in a field where occupational diseases are as numerous as the number of pathogenic bacteria. Only by being careful with your techniques can you hope to prevent injury to yourself and your co-workers.

5. Sterilization and Disinfection

5-1. Bacteriological identification requires that pure cultures of microorganisms be studied. Since microorganisms are everywhere, all materials that you use to cultivate bacteria must be sterile. It is important that you, as a bacteriology technician, know the methods of removing, killing, or preventing (inhibiting) the growth of microorganisms. The many different kinds of organisms vary in the manner by which they can be destroyed. Therefore, no single method of sterilization is suitable for all organisms or all situations. Certain facts can guide you to select the best method of sterilization.

5-2. **Sterilization Fundamentals.** There are four basic reasons for sterilizing materials:

- To prevent infection of man.
- To prevent contamination of materials used to obtain pure cultures.
- To prevent interference by contaminating microorganisms in those processes which require pure cultures.
- To prevent spoilage due to bacterial contamination.

5-3. There are three generally accepted methods of sterilization:

- By physical agents such as heat and ultraviolet radiation.
- By mechanical (filtration).
- By chemical agents.

5-4. *Common terms of sterilization.* We will discuss definitions of common terms, the principles of methods of sterilization, methods of sterilization, standardization of disinfectants, and aseptic techniques. Let us begin with terms associated with sterilization. It is essential for you to know the terminology if you are to use the various agents correctly for the purpose of sterilization.

5-5. *Sterilization* means the complete destruction or removal of all living forms of microorganisms. By living forms, we mean the spore or resting phase of the organism as well as the vegetative form.

5-6. A *disinfectant* is a chemical agent which will kill microorganisms. This does not necessarily mean sterilization, although some methods of disinfecting can also sterilize. Generally, disinfectants will kill the living vegetative forms of microorganisms, but not the heat-resistant spores. Synonyms for disinfectant are germicide and bactericide, both of which are used quite extensively.

5-7. An *antiseptic* is a substance which can inhibit the growth of microorganisms without actually killing them. Antiseptics are generally used to inhibit organisms which came in contact with the body. Although the terms "bacteriostasis" and "antibiotic" have the same definition as antiseptic, they have a different significance.

5-8. The suffix of bacteriostasis, *stasis*, means "to stand still." Bacteriostatic agents do not cause the immediate death of microorganisms, but instead prevent multiplication. The microorganisms will eventually die without a significant increase in their numbers. Good examples of a bacteriostatic agent are low temperature, desiccation (of some organisms), and antibiotics.

5-9. An antibiotic, literally translated, means "antigrowth substance" or "growth inhibitor." Its effects are similar to bacteriostasis and, in fact, act as a bacteriostatic agent.

5-10. In a previous paragraph we referred to sterilization as being the complete destruction of bacterial life. Different bacteria are affected in different ways, so let's discuss these effects.

5-11. *Viability of organisms.* Viability means "ability to live." To remain viable, organisms must be able to propagate over an extended period when placed in a suitable environment. To do this, bacteria must carry out the vital processes of life. They need water to maintain the proper cytoplasm consistency and osmotic stability. They need food, oxygen or carbon dioxide, a proper temperature, and the correct acid-base environment. If these are available, the metabolism of the organism takes place within the cell and multiplication results. Removal of the water or altering the physical conditions that control the chemical metabolism can cause the organism to die. There are, however, certain bacteria which can survive this alteration. To destroy them, it is necessary to act on their internal metabolic processes.

5-12. The variations in susceptibility of microorganisms to various methods of sterilization are very important when choosing a means of sterilization and the conditions under which sterilization can take place. There are essentially six considerations to be investigated:

- Related genus.
- Growth curve (stage of organism).
- Presence of organic matter.
- Temperature.
- Time.
- pH.

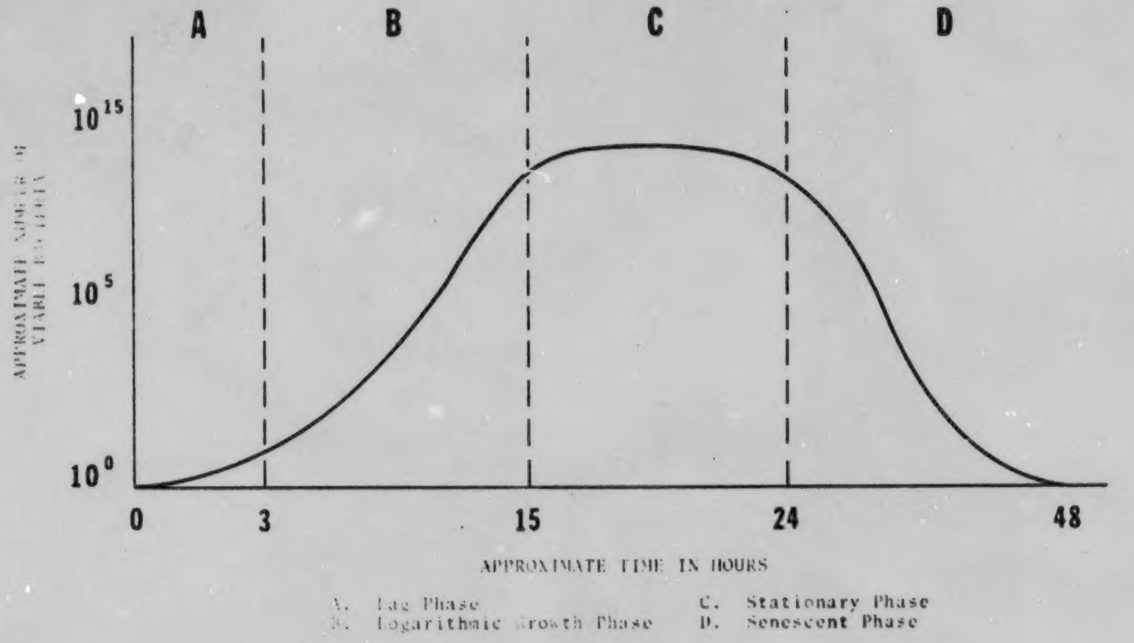


Figure 5. Typical growth curve of a bacterial culture.

5-13. As you will learn in a later chapter, a genus of bacteria refers to a group of related organisms. A simple example may help to explain this now. The organisms, *Staphylococcus* and *Bacillus* have enough morphological and biochemical behavior differences to belong to separate genera. The biochemical behavior between genera is at times sufficiently different to warrant the selection of a different means of sterilization. The main difference existing between genera which affects the choice of sterilization methods is the organism's ability to produce spores. Spore producers are much harder to destroy in the spore stage than in the vegetative stage. *Bacillus* is a spore-former; *Staphylococcus* is not. *Bacillus* needs to be sterilized by heat and pressure (autoclaving).

5-14. All microorganisms go through four phases during their life cycles or growth curves. They are:

a. Lag phase. This the period of time it takes the organism to become acclimatized to its new surroundings. Multiplication has not yet begun or is minimal.

b Logarithmic phase. In this phase the bacteria reproduce at a logarithmic rate, that is, one becomes two, two becomes four, four becomes eight, etc. The colonies increase in size and gain their characteristics during this stage.

c. Stationary phase. At this point of growth there are about as many bacteria dying as there are being produced. The number of viable organisms have reached a maximum.

d. Senescent or death phase. More bacteria are dying than are being produced. Eventually all will die because of the lack of nutrients and a change in pH due to waste products.

5-15. During the first two phases and through part of the third phase the organisms are young and growing. Their enzymatic functions are fairly rapid and their cell walls are vulnerable to the effects of the various sterilization techniques. As the bacteria become old, their functions slow down and those that can, begin to go into a resting stage. At this point, killing the organism becomes more difficult. It is easier to destroy a young growth of bacteria than an old one. You will easily recognize which phase the bacteria are in as you work with cultures. Notice the four phases shown in figure 5. It will be easy for you to select sterilization and decontaminating procedures.

5-16. Most disinfectants have a general affinity for the protein and protoplasm of the bacteria. Consequently, the presence of extraneous quantities of blood serum, mucus, or feces may protect the organisms by combining with the disinfectant before the disinfectant has a chance to affect the bacterial cell. Because of this, culture growths are easier to sterilize than body fluids.

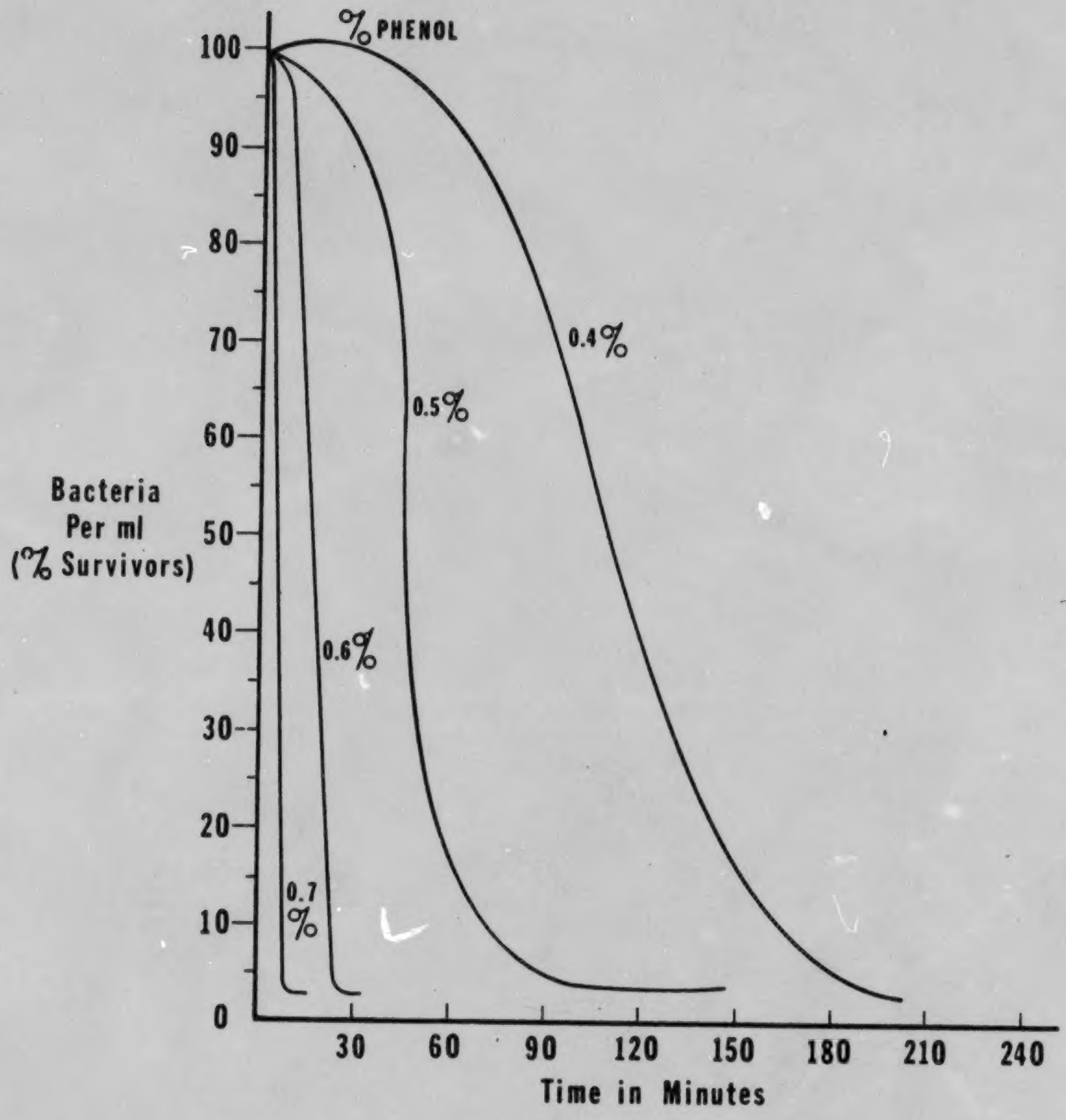


Figure 6. Chart—concentration vs affect.

5-17. No routinely used disinfectant acts immediately. It takes time for a disinfectant to act and this depends upon several factors, four of which are:

- Concentration of organisms.
- Concentration of disinfectant.
- Temperature.
- Presence of cells in the culture having varying susceptibilities.

5-18. The true concentration can refer to the dilution of the disinfectant or to the number of organisms in the culture. The more concentrated the disinfectant (within limits), the more effective it will be. The relation of concentration to effectiveness is shown in figure 6. The concentration of most disinfectants is stated on the label. You should always follow the manufacturer's instructions for using and diluting any disinfectant. The concentration at which a disinfectant is used determines how effective it will be. Any disinfectant has an optimum dilution where it will do the best job.

5-19. The warmer the disinfectant, the more effective it is and the quicker it will react. A higher temperature generally increases a disinfectant's effectiveness by decreasing the viscosity, increasing the acidity of the bacterial surroundings, and lowering the bacterial surface tension. Heating too much can decrease the disinfectant's effectiveness by diminishing adsorption to the cell wall. We can say that the temperature effect in this type of reaction is similar to that created in a chemical reaction: the temperature increases in arithmetic progression, while the speed of the reaction proceeds in a geometrical progression. The temperature and reaction speeds are diagrammed in figure 7. Keep in mind, however, that high temperatures may destroy the disinfectant, therefore, heating disinfectants should be done carefully.

5-20. Increasing the acid or alkaline (basic) properties (pH) of the disinfectant increases the effectiveness. Heat, as used for sterilization, increases the acidity of the solution surrounding the cells. This change in pH affects some of the metabolic processes of the bacterial cell. As a result, the bacteria may die.

5-21. **Physical Agent Sterilization.** There are many physical means of killing or inhibiting the growth of microorganisms. Methods in common use include:

- Moist heat.
- Steam under pressure.
- Dry heat.
- Incineration (open flame).
- Ultraviolet radiation.
- Mechanical (filtration).

In the following paragraphs, we will discuss each of these methods separately and give you a working knowledge of the principles on which they work.

5-22. *Moist heat.* Moist heat (boiling in water and steam) will coagulate the protein within the bacterial cell. This occurs in two stages: (1) the water reacts with the protein (denaturation) and (2) the altered protein separates out as particles (flocculation). Coagulation of the protein is directly related to time and temperature. Different genera of microorganisms vary in their susceptibility to heat and no one temperature can cause the death of all genera.

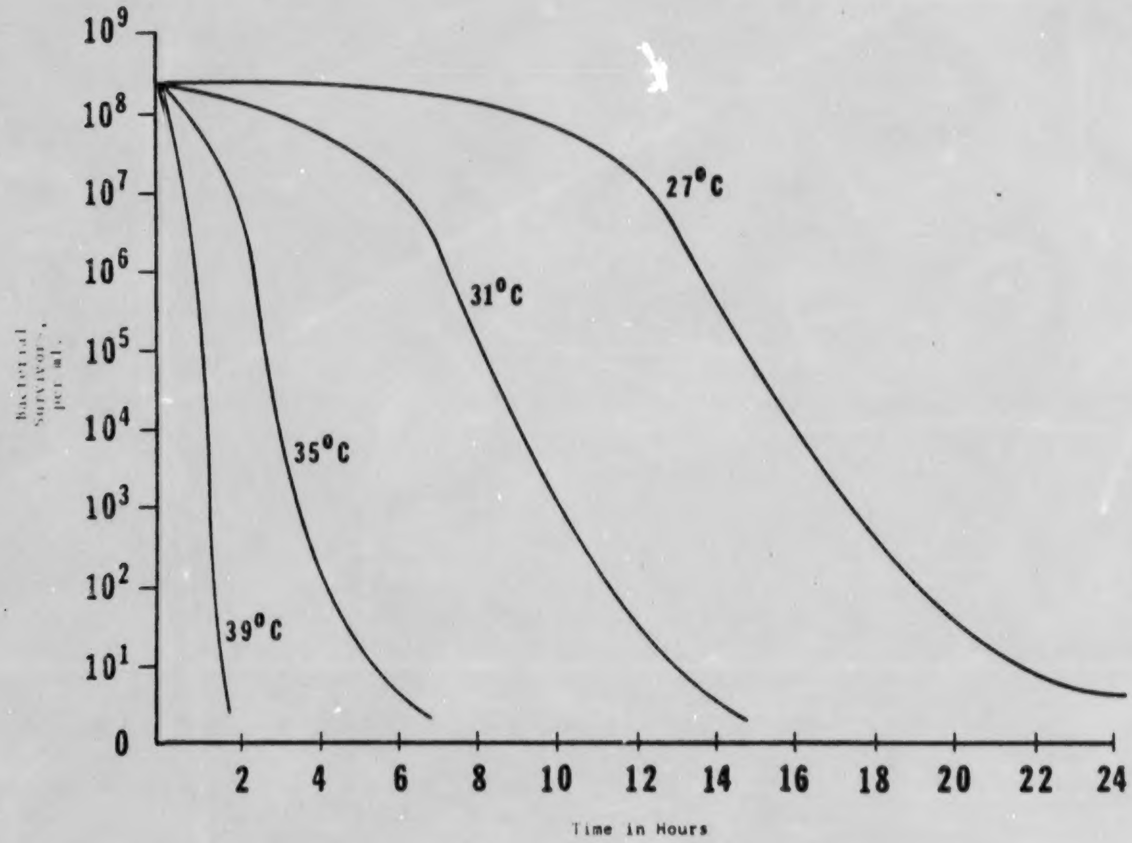


Figure 7. Temperature vs disinfectant affect.

5-23. Using boiling water for disinfecting purposes is a simple procedure. You can use it almost any place where water and a container to boil the water are available. Boiling is a good method for disinfecting; however, it can never be depended upon for complete sterilization, especially at elevations above sea level. Ordinarily a 5-minute boiling procedure in New York City, which is at sea level, would be sufficient to sterilize water. However, it would take a much longer time to sterilize water at Tahoe, Nevada, where the altitude is 6,225 feet.

5-24. If boiling is used for sterilization, as might be the case with outpatients for sterilizing their own containers, sufficient time must be allowed to compensate for the difference in altitude and the change in the boiling point. Remember also that spores may remain alive after hours of boiling. Use boiling as a means of sterilization under carefully controlled conditions.

5-25. The inspissator is another means of moist-heat sterilization. Certain materials are altered or completely destroyed at a temperature of 100° C. For example, blood serum is not suitable for use in instances where it has been subjected to temperatures above 90° C. An inspissator will allow the temperature to rise slowly between 80° C. and 90° C. Most laboratories do not have facilities or requirements to warrant the use of an inspissator. It is also time consuming. It is best to purchase any medium which requires inspissation directly from a commercial concern. When received it is usually ready for use.

5-26. *Steam.* The use of moist heat in the form of steam, for the purpose of sterilization can be divided into two groups.

- Free-flowing steam.
- Autoclaving (compressed steam).

5-27. To get *free-flowing* steam, you use a covered container to hold steam without building up pressure. You should remember that free steam, as with boiling water, does not reach a temperature above 100° C. or 212° F. In higher elevations the temperature would be even less.

5-28. A British scientist, Tyndall, noticed that after boiling and standing for a period of 24 hours at room temperature, the dormant and heat-resistant spores in a solution would germinate and grow. Reboiling would then kill these forms.

5-29. The Arnold Sterilizer uses this principle. Expose the material to be sterilized to flowing steam for 15 to 30 minutes on 3 consecutive days in the sterilizer. Each day after steaming, the material is allowed to cool and incubate; this allows the spores to germinate into the vegetative form. After the third steaming, the material should not contain viable spores and is considered sterile.

5-30. Fractional sterilization (Tyndallization), using an Arnold Sterilizer, is a practical means for sterilizing materials which can be injured by excessive heat. You may sterilize certain media, such as gelatin, sugars, and potatoes used for bacteriological procedures by this procedure.

5-31. The autoclave will kill microorganisms, including heat-resistant spores, with moist heat at temperatures above that of boiling water. Water boils when the vapor pressure is equal to the pressure of the surrounding atmosphere. Hence, if the pressure is increased in a sealed vessel, the boiling point will rise above 100° C. In a closed system, the exact temperature at which water will boil depends upon the pressure employed. Since it is not the pressure, but the temperature and moisture which are the effective lethal agents, the autoclave is equipped with a thermometer as well as a pressure gauge. The exposure time should be based on the temperature reading rather than the pressure reading, because air trapped in the chamber may cause the pressure to build up before the desired temperature is reached. The maximum temperature corresponding to any given pressure can only be obtained when the interior of the vessel is saturated with steam. In table 2, the temperature attained at different pressures of saturated steam is given. Only culture media, solutions, or materials which are not destroyed or altered by excessive heat should be subjected to autoclave sterilization.

5-32. To achieve the desired results through autoclave sterilization, you must observe the following principles:

a. The autoclave must be in proper operating condition and should be periodically evaluated. The following cleaning procedure should be used to insure proper functioning.[1]

Step 1 (daily procedure): Wash inside of sterilizer, using Calgonite solution. Never use strong abrasives, steel wool, etc. A long-handle cellulose sponge mop is helpful in cleaning the longer chambers. (Chambers should be cool before cleaning).

Step 2: Remove plug screen from bottom of chamber and free its openings from lint, sediment, etc.

Step 3 (weekly procedure): Wash sterilizer loading car, using Calgonite. Remove plug screen and flush chamber drainline with hot solution of trisodium phosphate—1 ounce (2 tablespoonfuls) to 1 quart of hot water. Follow with a flush rinse of 1 quart of tap water.

b. The autoclave should never be overloaded.

c. Tubes should be packed loosely in baskets or racks and never placed in containers capable of trapping air.

---

[1] American Sterilizer Company, Erie, Pa.

TABLE 2
PRESSURE TEMPERATURE RELATIONSHIPS IN THE AUTOCLAVE

Temperature in Degrees Centigrade	*Steam Pressure in Pounds*
108	5
116	10
121	15
127	20
131	25
134	30

d. Large quantities of media in single containers should be preheated to avoid undue time lag. If possible, distribute media to several smaller containers to affect complete heating and sterilization.

e. All air must be exhausted from the autoclave and the temperature reached before timing the sterilization cycle.

f. Media should never be overautoclaved or reautoclaved.

g. Flasks and tubes should never be filled to more than two-thirds capacity (one-half capacity is better).

h. All media should be removed from the autoclave as soon as possible after sterilizing.

i. An autoclave temperature of 121° C. at 15 pounds pressure for 15 minutes or longer is required for the sterilization of most of the materials routinely used in the bacteriology laboratory. Figure 8. illustrates the parts of the autoclave and the flow of steam through the autoclave.

5-33. *Dry heat.* This method is used when direct contact of saturated steam to all surfaces of a material is impractical. We use an electrically heated, thermostatically controlled oven to sterilize glassware and metal utensils.

5-34. When loading the dry-heat sterilizer, observe the following rules:

- Do not load the chamber to capacity.
- Do not pack containers close together. Allow space between them so that the hot air can circulate.
- Do not stack articles against side walls. The hot air must circulate freely.

5-35. The requirements for dry-heat sterilizing depend on the characteristics of the various items being sterilized. As a general rule, however, the following schedule is offered:

- 172° C. for 1 hour.
- 160° C. for 2 hours.
- 121° C. for 6 hours or longer.

The above times and temperatures include a reasonable time allowance for temperature change during the exposure time. All items to be sterilized by the dry-heat method must be thoroughly cleansed before exposure. Otherwise, heat will fix the grease, dirt, and other material to the equipment.

5-36. *Incineration.* Incinerating is a method to assure complete destuction of contaminated materials. You should use common sense when deciding what may be burned, and take caution to assure complete burning of the discarded material. Always observe local practices in the use of the incinerator.

5-37. *Ultraviolet radiation (open flame).* This method of sterilization has come about after prolonged investigation. There is no doubt that light and electomagnetic rays of short wavelength exert an effect when absorbed by the bacteria. Its use is limited, however, in that it must be applied directly to the surface being sterilized. It cannot penetrate glass, and exposure must be prolonged. Eyes must be protected from the rays. It can be used to sterilize rooms in a hospital, such as the operating room. It is quite effective in killing microorganisms on the floor, walls, instrument ta-

33

MODIFICATIONS

Page 22 of this publication has(have) been deleted in adapting this material for inclusion in the "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education." Deleted materials are copyrighted and could not be duplicated for use in vocational and technical education.

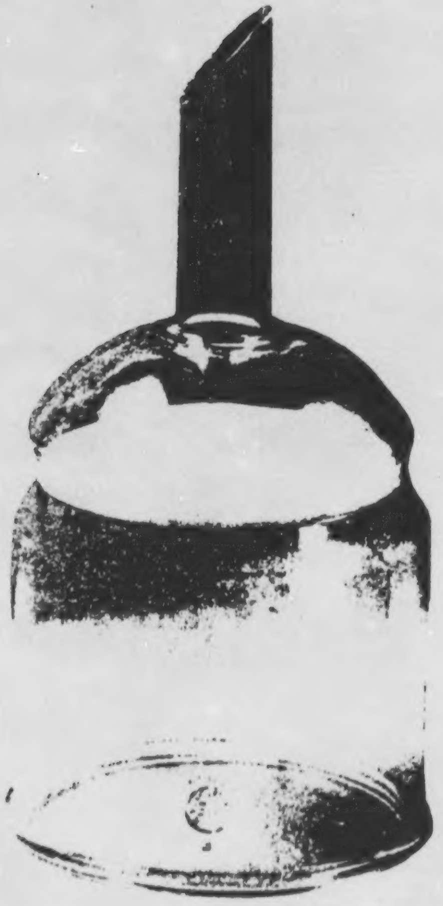


Figure 9. Pyrex fritted glass filter.

bles, and in the air. The treatment of certain biological materials, such as vaccines and plasma, to kill any contaminating viruses is becoming common practice. In every case, it is the radiation and energy absorbed by the bacterium which bring about its destruction, and not the energy applied.

5-38. *Mechanical (filtration).* Filtration is a mechanical means of sterilization. It removes rather than destroys microorganisms from the material to be sterilized. Certain types of media are destroyed or altered by heat. Materials such as carbohydrates, serum, ascitic fluid, urea, potassium, and tellurite are thermolabile. These substances in solution are passed through a filter of fine porosity to remove all bacteria. Examples of different types of filters are listed below.

a. Berkfield filters are manufactured from infusorial earth. They can be obtained in three porosities, namely, the *V* (coarse), *N* (normal), and *W* (fine). Only the *N* and *W* filters of this type should be used for sterilization.

b. Chamberland filters are made of unglazed porcelain in graded porosities designated *L*–1, *L*–2, *L*–3, etc. The filter gradations ranging from *L*–5 to *L*–7 are bacteria proof.

c. Pyrex brand filters consist of pulverized glass (fritted ware) available in *UF* or ultrafine porosity for bacterial sterilization. This type is shown in figure 9.

d. Seitz filters consist of compressed asbestos pads of the *K* (germicidal) porosities, as shown in figure 10. All the above filters, except the Seitz, are permanently mounted in a funnel. The Seitz has filter pads which are clamped in the base of a metal funnel.

5-39. Clean the Berkfield filter by successive boiling in 2 percent sodium carbonate and distilled water, then rinsing with water. Pyrex filters are cleaned by immediately flushing with water under pressure, placing in hot, hydrchloric acid, and thoroughly rinsing with distilled water. Clean Chamberland or porcelain filters in standard chromic acid washes, flush thoroughly with water under pressure, and rinse with distilled water. The Seitz filter pads are disposable. You must wash the metal funnel parts in a detergent and rinse thoroughly.

5-40. The sterilizing effect of filters is achieved mostly by adsorption of the microorganisms to the surface of the filter and filter funnels rather than by sieve action. All equipment used for filtration must be sterilized prior to use. The filter will absorb those microorganisms from the fluid coming through the filter but, naturally, cannot remove those organisms below the filter. Figure 11 is an example of the setup used for filtration.

5-41. Another means of filter sterilizing is a filter attachment on a syringe. This allows the filter sterilization of small amounts of fluids. One example of this equipment is that produced by the Millipore Filter Corporation. Individual syringes with the filter attachment are autoclaved and stored in a sterile condition until needed. You fill the syringe by removing the plunger and pouring the fluid into the barrel. Insert the plunger and apply steady pressure. This will force the fluid through the filter into a sterilized tube or tubes. This method offers the advantage of using less medium and will save on storage space and resources.

5-42. **Chemical Sterilization.** There are three sites in the bacterial cell that are susceptible to the action of chemical agents. These are surface layer, nuclear material, and enzyme systems.

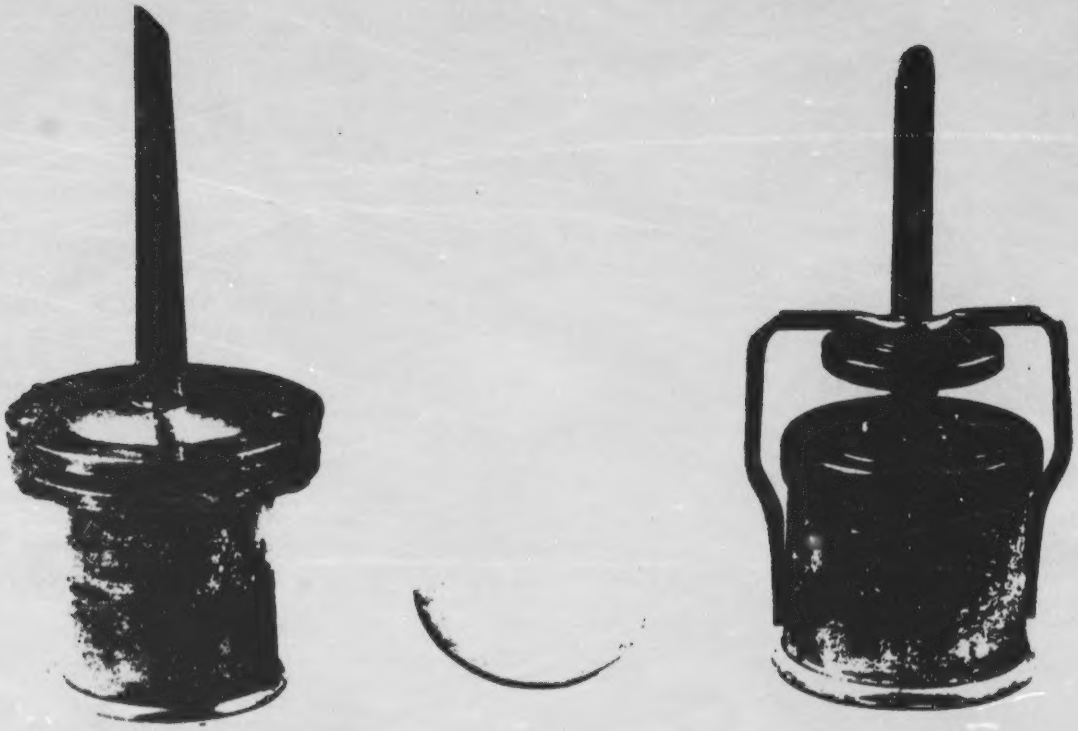


Figure 10. Seitz filter.

Chemical agents perform their disinfecting and sterilizing action on these sites by coagulation of proteins as surfactants, by specific chemical combinations, and by nonspecific chemical combinations. Action by chemical agents may be by one of more of the above methods. A brief discussion of each of these methods follows.

5-43. *Coagulation of proteins by surfacts.* The coagulation of proteins is similar to that of cooking an egg. The protein (albumin or white) of the egg coagulates and turns white. Any agent which can induce coagulation is lethal to a living cell.

5-44. Surfactants are chemical agents which coat the surface of the organism and/or the surface of certain enzyme systems to prevent the microorganism from absorbing or utilizing nutritional substances. This will cause the organism to die. Certain chemical agents injure or destroy the cell wall, causing immediate lysis of the cell or changes in the cell membrane permeability. Polymyxin, an antibiotic, works in this manner.

5-45. *Specific chemical combinations.* In low concentrations, these enter the cell of the microorganism and stop or interfere with enzyme groups. This will lead to bacteriostasis or death depending upon the type of cell and chemical agent. The sulfonamide drugs and many antibiotics are effective in this manner.

5-46. *Nonspecific chemical combinations.* These chemicals will combine indiscriminately with the protein and other compounds within a bacterial cell. These substances are nonspecific in their action and will combine readily with feces, blood, body tissue, and mucus as well as the protein of the microorganisms. Chlorine, iodine, formaldehyde, and phenol are examples.

5-47. **Chemical Agents.** Now that you have a brief understanding of the way in which chemical agents affect microorganisms, we will discuss these chemical agents:

- Halogens (oxydizing agents).
- Salts.
- Heavy metals.
- Synthetic detergents.
- Organic solvents.
- Dyes.
- Gaseous agents.
- Phenols.

5-48. *Halogens.* Halogens used as disinfecting agents are chlorine, iodine, and bromine.

These are used frequently and are known to be effective disinfectants.

5-49. Chlorine in the form of calcium or sodium hypochlorite has a high bactericidal property. It is commonly purchased as a 5 percent bleach solution in most grocery stores. Although chlorine is quite effective as a disinfectant, it has certain disadvantages which we must consider before using it. These are:

(1) It is readily neutralized by protein and other organic material (nonspecific).

(2) It is poisonous.

(3) It tends to evaporate from the container.

(4) In aqueous solution it is unsuitable for polished surfaces as it does not spread evenly over the surface.

(5) It attacks metals.

Chlorine is probably the most effective agent for use in disinfecting water. It can be used as a 1.0 percent solution for cleaning hands; however, the hands may develop a bad odor. (A thiosulphate solution will remove the odor.)

5-50. Iodine is the most active halogen in its antimicrobial effect. As a 1.0 percent solution in 70 percent ethanol or in a potassium iodide solution it is a good disinfectant for the skin. However, iodine is not used very often for large-scale disinfecting procedures, because it has most of the disadvantages of chlorine. Solutions of iodine combined with a surfactant carrier (iodophor) are available commercially and may be used for a variety of purposes, including sanitizing dishes and sterilizing thermometers. Examples of products available commercially are are Wescodyne, Iodide, and Betadins.

5-51. Bromine, although as effective as chlorine, it is very irritating to the exposed body surfaces. It offers no advantages over chlorine and is infrequently used.

5-52. *Salts.* The mechanism by which a salt solution kills microorganisms is unknown. It does have a definite effect on bacteria, especially when mixed with a proper amount of calcium salt. Although not used often as a disinfectant, salts can be used to help the bacteriology technician prevent the growth of certain genera of microorganisms. With this knowledge, we can reduce the work necessary to identify organisms which will grow in broth with a high concentration of a salt such as sodium chloride.

5-53. *Heavy metals.* Compounds of the heavy metals mercury, silver, and copper have a def-

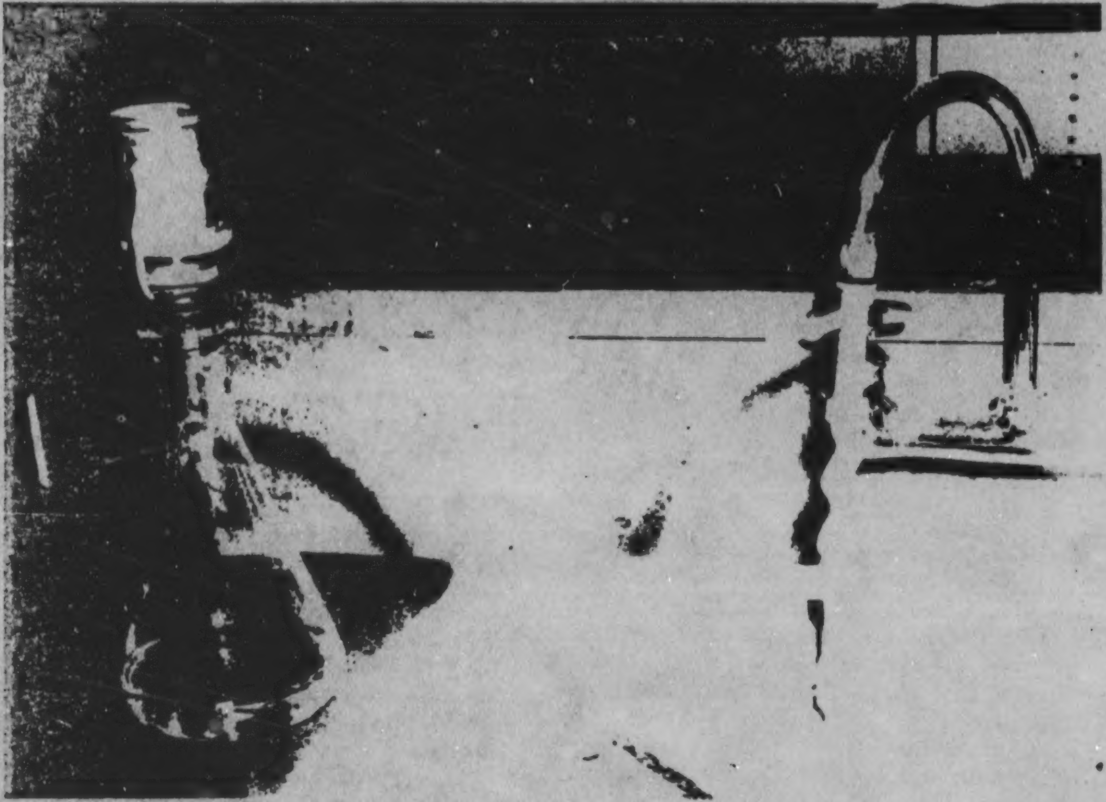


Figure 11. Filter attached to suction.

inite but limited use as disinfectants. Their action is more bacteriostatic than bactericidal. In high concentrations they act on the bacterial cell to coagulate the protein, but in low concentrations they interfere with certain enzyme systems.

5-54. The more common heavy metal compounds in use include mercury bichloride, copper sulfate, and silver nitrate. Merthiolate (Thimerosol), Mercurochrome, Argyrol, and Protargol are familiar names for commercially prepared solutions of the compounds of mercury and silver. Solutions of copper sulfate are used mostly as fungicides.

5-55. *Synthetic detergents.* These are often used for sterilization. The word "detergent" is derived from Latin and means "to cleanse." Detergents including household detergents are strong, surface active agents. They are essentially nonirritating to raw surfaces, and in dilutions varying from 1/100 to about 1/15000, destroy vegetative forms of bacteria. Some of the synthetic detergents will precipitate various proteins, agglutinate bacteria, and are toxic to white blood cells (leukocytes). For vegetative forms of microorganisms on the skin, detergent action is more bacteriostatic than bactericidal. Their action is decreased by the presence of organic matter and saline, but not by distilled water. Generally, the Gram-positive bacteria are affected more readily than the Gram-negative organisms. Detergents are essentially of three types—anionic, cationic, and nonionic.

5-56. Anionic detergents consists of soaps and fatty acids which yield a negatively charged ion when mixed with a suitable solvent. This type of detergent is more active at an acid pH and is more effective against Gram-positive organisms. Its lack of effectiveness against Gram-negative organisms is thought to be due to lack of phospholipid material at their surface. When the detergent reacts with this phospholipid in Gram-positive organisms, salts and formed and death results.

5-57. The cationic group of detergents has a water-soluble group of particles which will dissociate to form positively charged ions. These ions will be absorbed on the surface of the negatively charged bacteria, causing a loss of semipermeability with a resultant leakage of phosphorous, nitrogen, and other substances important to bacterial metabolism. Cationic detergents are more active at an alkaline pH. Zephiran and hexachlorophene are examples of cationic detergents.

5-58. Nonionic detergents do not ionize or dissociate in water and are therefore pH independent. They are relatively nontoxic and are often used as additions to commercial detergents. Tween 80, which may even facilitate bacterial growth, is an example of a nonionic detergent.

5-59. You cannot use synthetic detergents at random. Your choice must be made intelligently so that maximum benefit can be achieved. Do not mix anionic and cationic detergents as the negative and positive charges will combine and neutralize each other and cancel out the antiseptic or disinfectant effect desired.

5-60. *Organic solvents.* Alcohol, the most used of the group known as *organic solvents,* is a good example of this type of disinfectant. You already know that ethyl alcohol (ethanol) has an optimum disinfectant quality at a strength of from 50 to 70 percent. A point to remember is that this percentage refers to the final dilution of alcohol. To illustrate—to disinfect dry bacteria, a solution of 50 to 70 percent ethanol is best and can be used directly; but if you wish to disinfect a tube of broth containing viable organisms, it is best to add absolute ethanol to the broth so that a final dilution of the alcohol in the broth represents 50 to 70 percent. The addition of dilute alcohol to the broth tends to dilute the alcohol further and, as a result, it is less effective. To disinfect the hands, 70 to 80 percent alcohol is best if the hands are dry, and 80 to 96 percent alcohol if the hands are wet.

5-61. Ethyl alcohol in the proper strength works well to destroy the vegetative stage of bacteria. It takes longer to kill dry bacteria than wet bacteria. To destroy spore forms, the addition of sulfuric acid to 70 percent alcohol (1.0 percent final volume of sulfuric acid) works effectively. You can use also sodium hydroxide instead of sulfuric acid.

5-62. Isopropyl alcohol (80 percent) is useful as a skin disinfectant. It is a better fat solvent and more bactericidal than ethyl alcohol. Both have the disadvantage in that they are rendered rather inactive by the presence of mucus or pus. In this respect, to be effective in disinfecting thermometers, you must wipe them clean before placing them in the alcohol solution, or in any disinfectant solution where its action is based on the coagulation of protein.

5-63. *Dyes.* There is very little known about the relation between the biological activity of microorganisms and the chemical structure of *dyes* used as disinfectants. It has been observed, however, that certain genera of bacteria are more sensitive to some dyes than are other genera. Although dyes are not generally used as sterilizing or disinfecting agents, they are put to good advantage for the isolation and identification of bacteria by incorporating certain dyes into various culture media. Crystal violet, which will stain both staphylococci and streptococci in the Gram stain, can be diluted so as to be effective in separating streptococci from staphylococci by preventing the growth of the staphylococci. We will discuss individual dyes with the medium concerned in the chapter on media.

5-64. *Gaseous agents.* These agents include formaldehyde, sulfur dioxide, chlorine, ethylene oxide, and beta-propiolactone. Each has certain limitations and most clinical laboratories have little use for gaseous agents as a means of sterilization.

5-65. Formaldehyde is used very little. It has a very low penetrating ability and is affected by the presence of protein matter. It is most effective in destroying vegetative forms of bacteria in a concentration of 1 to 2 mg. per liter of air at 80 percent relative humidity. Its chief advantage is in the disinfecting of smooth surfaces. To penetrate and disinfect blankets and the like, the formaldehyde should be mixed with steam. You should not use formaldehyde on good furniture, as the formaldehyde will convert to paraldehyde and form a white film which is hard to remove. Formaldehyde cannot be relied on to destroy spores or acid-fast bacilli.

5-66. Sulfur dioxide and chlorine are not often used because they are too poisonous. However, destructive heat and aqueous solutions do not have to be used with sulfur dioxide and chlorine. As gases, they can be applied in large volumes to rooms. Sulfur dioxide and chlorine will convert to the acids sulfurous and hypochlorous, respectively; both are corrosive. A good cleaning is necessary after their use.

5-67. You will come in contact with material sterilized by the gaseous agent *ethylene oxide* Practically all disposable, plastic materials are sterilized by this gas. Ethylene oxide is very effective and kills bacterial spores. The gas is highly explosive at certain concentrations and its use is generally limited to a closed system similar to an autoclave. It has an advantage over formaldehyde in that ethylene oxide is highly diffusible and can be used to sterilize fabrics, catheters, cystoscopes, and other equipment.

5-68. Beta-propiolactone is a good sterilizing agent in that it kills both the vegetative and spore forms of microorganisms. It acts by combining with the sugars, cellular proteins, and fats of bacteria. It can be used to sterilize tissues for grafting and for sterilizing vaccines which could not be sterilized in any other way. It does not leave a residue as does formaldehyde. It is several thousand times more active than ethylene oxide.

5-69. *Phenol and cresol products.* Phenol (carbolic acid) and creosol products are obtained by the destructive distillation of coal tar. Phenol and its many derivatives are surfactants. They probably act by forming an insoluble albumin compound when in contact with protein, or by coagulating the protein of the cytoplasm by penetrating into the organism as a colloid. They have a sterilizing effect in that they can kill both the vegetative and spore forms of bacteria. Creosols mixed with green soap are sold under the trade names Lysol and Creolin and are four and ten times more effective than phenol, respectively. The addition of sodium chloride to the commonly used 5 percent solution of phenol increases its effectiveness. The addition of ethyl alcohol reduces its effectiveness considerably. Certain of the coal tar derivatives known as bis-phenols are incorporated in solution with other surfactants or detergents to produce the surgical soap and hand wash Gamophen and Phisohex.

5-70. **Characteristics of a Good Disinfectant.** To be effective a disinfectant must possess the following qualities:

a. Solubility.
b. Stability.
c. Noncorrosiveness.
d. Nonbleaching.
e. Effective power of penetration.
f. Ease of application.
g. Cheap in cost.
h. Nontoxic to animals.
i. High coefficient of disinfection.
j. Deodorization.

All of the above qualities, with the possible exception of high coefficient of disinfection, are easy to understand in that you use them everyday. We discuss this quality in the following paragraphs.

5-71. To test the relative efficiency of disinfectants, some disinfectant had to be selected as a standard and all others tested in relation to this standard. The disinfectant chosen was phenol (carbolic acid).

5-72. Principles which govern the estimation of the disinfecting power must be strictly adhered to.

a. The media used in all cases of standardization must be alike in composition and pH.

b. The organism used must always be grown and treated under the same conditions.

c. Temperature should remain constant.

Although the standard methods employ constant factors as listed above, you must remember that even though a high proportion of organisms are killed before sterilization is complete, a few organisms may resist the disinfectant action for a long period of time.

5-73. *Phenol coefficient method.* A method which has been used for a long time and which appears to be suitable for most purposes is called the phenol coefficient method. This method tests the effectiveness of an unknown disinfectant only if conditions are carefully controlled.

5-74. There are in general use several methods of determining the phenol coefficient of a disinfectant. Three of these are:

a. The U.S. Hygienic Laboratory method.
b. The Rideal-Walker method.
c. The Food and Drug Administration method.

The Food and Drug Administration method is generally the method used in testing many of the disinfectants on the market. The method compares the ability of phenol against an unknown disinfectant, when diluted to kill the Hopkins strain of *Salmonella typhosa.* The figure representing the dilution of phenol that kills the organism in 10 minutes is divided into the dilution figure of the unknown disinfectant which killed the organism in 10 minutes. For an illustration: Phenol at a dilution of 1:100 killed the organism in 10 minutes. The unknown disinfectant killed the organism in the same time, but at a dilution of 1:250. Dividing 100 into 250 gives a phenol coefficient of 2.5.

5-75. The significance of the phenol coefficient has limitations. For example, a disinfectant may be found to have a phenol coefficient of 40 when tested in distilled water. The compound may be quite ineffective when applied in the presence of organic matter, pus, saliva, feces, or milk. Then again, when tested against some other organism such as *Staphylococcus aureus* it may have a much lower coefficient. Disinfectants are often diluted to offer a concentration roughly 20 times the phenol coefficient. This "use dilution," though considered unreliable, is the dilution in which disinfectants are used.

5-76. *Sterilization guidelines.* To aid you in your use of disinfectants and sterilization techniques, you can follow these guidelines:

a. The vegetative form of the microorganisms is easier to kill than the spore form.

b. Spores and acid-fast bacilli are best killed by heat. (Liquid disinfectants are fairly ineffective because of the lipid material surrounding the acid-fast bacilli.)

c. Bacteria suspended in a nonprotein-type medium are easier to destroy than those suspended in a protein-type medium. It is more difficult to destroy bacteria in protein-type media if the media are also nutrient in nature.

d. The addition of an acid or a base to protein media increases the effectiveness of the disinfectant. A given concentration of H^+ ions is more effective than the same concentration of OH^- ions.

e. The sterilization process can be increased by raising the temperature of the disinfectant.

f. Dissolving certain germicidal chemicals in alcohol may either decrease or increase the effectiveness of the solution.

g. The presence of organic matter usually lowers the effectiveness of liquid disinfectants.

h. An emulsified disinfectant and/or a liquid disinfectant containing a wetting agent is best for sterilizing smooth surfaces.

i. Powder disinfectants must be placed in solution to be effective, otherwise, they act only as deodorants.

5-77. **Asepsis Testing.** Asepsis is briefly defined as the absence of septic matter, or freedom from infection. As a bacteriology technician, you will be testing the effectiveness of hospital aseptic techniques and practice aseptic techniques in much of your routine daily work. This section will deal primarily with the laboratory's responsibility (consequently yours) toward preventing the spread of hospital-borne diseases.

5-78. There are many behind-the-scene activities in a hospital. One of these is the prevention of the spread of disease within the hospital. There are many different facets to this particular activity, and you will be required to lend support to this extremely important program. The Hospital Infection Committee with the approval of the commander, makes recommendations for the prevention and control of infections. The following paragraphs will inform you about aseptic techniques and in-house infections.

5-79. *Sterility testing.* This refers to those procedures which test the effectiveness of the various methods of sterilization, more specifically, the autoclave and room disinfection.

5-80. You can use the autoclave to sterilize medical equipment. To assure sterility of this equipment, it must be tested at regular intervals. You can do this testing in three ways.

(1) Testing the equipment itself by culturing.
(2) Using heat sensitive dye indicator strips.
(3) Using spore strips.

5-81. First, you must test the equipment. In this method, you open the packs containing the equipment. Swab the various articles using a sterile, cotton-tipped applicator and then place the swab into a suitable culture medium and incubate it. Check the broth for growth. A disadvantage of this method is that one can only assume that if no growth occurs from a particular article that specific article was sterilized by the autoclave. However, it is possible that the article

MODIFICATIONS

Page 29 of this publication has(have) been deleted in adapting this material for inclusion in the "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education." Deleted materials are copyrighted and could not be duplicated for use in vocational and technical education.

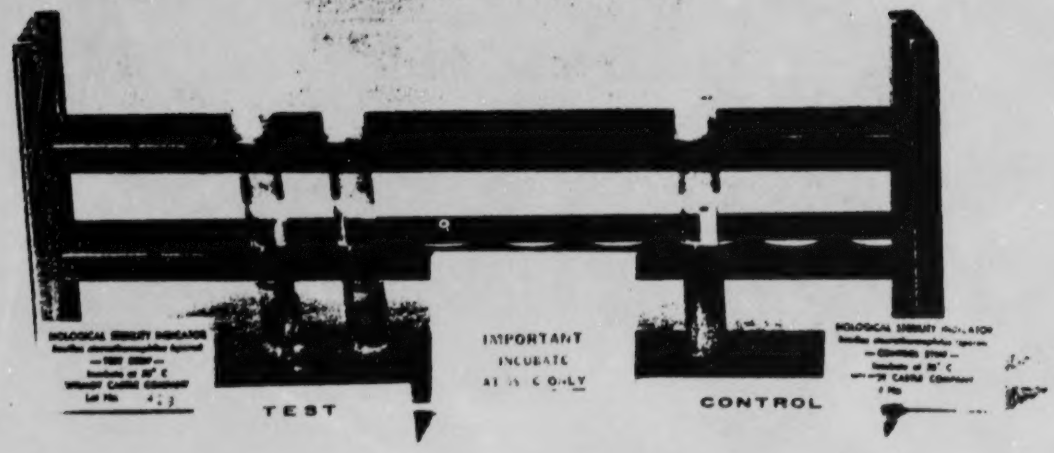


Figure 12. Biospore autoclave controls.

was sterilized when being cleansed. The other articles in the pack may not be sterile. This method is a fairly reliable substitute when heat-sensitive dyes or spore strips are not available. If the piece of equipment is small, you can place the entire article in a suitable culture medium to check for sterility.

5-82. If you are operating the autoclave, the use of a dye-indicator strip is a quick means to test the equipment for proper operation. The indicator is a thermolabile dye on paper strips, wrappers, tape, or it may be applied to thread or gauze. The dye turns a specific color if the contents have been sterilized at the correct tem-

CONTROL
TEST

Figure 13. Kilitampules.

perature for a sufficient length of time. You need only to observe these color changes to assure sterility.

5-83. You can make a more direct, though more time consuming, check on sterilization by the use of spore strips. A spore-impregnated strip of filter paper enclosed in a sterile envelope is placed inside the wrapper of the material to be sterilized. For autoclave testing, the spores of *Bacillus stearothermophilus* are most often used; and for dry heat and ethylene oxide the spores of *Bacillus subtilis (globigii)* are preferred. Table 3 shows the various time and heat combinations.

5-84. After sterilization, remove the sterile envelope and its spore strip and send them to the laboratory. Remove the strip from its envelope (using sterile forceps), place it in a suitable culturing medium, and incubate. If growth occurs within 7 days, sterilization has not been complete. The absence of growth indicates that sterilization has been complete. To assure yourself that sterility is the reason for the spores not germinating and growing, you must also use a control strip. This control strip is not autoclaved, and should grow organisms when cultured. You can see biospore controls in figure 12.

5-85. Sealed ampules containing culture media and spores of *Bacillus stearothermophilus* are also available on the market. After sterilization, the ampules are incubated at 55° C. As *B. stearothermophilus* is a strict thermophile, lack of sterility will be indicated by growth of the organism in the control ampule as shown in figure 13. As *B. stearothermophilus* is one of the most heat-resistant organisms known, its use offers a good indication of sterility.

5-86. *Testing of hospital rooms and furniture.* A large problem in hospitals is that of insuring complete disinfection or sterility of a room, or of hospital equipment used by a patient with a communicable disease. When the patient leaves the hospital, his room is cleaned and disinfected with one of the many agents suitable for this purpose. After cleansing, laboratory personnel test the room and its contents for sterility. This is usually done with sterile, cotton-tipped applicators by swabbing representative areas. The swabs are placed in suitable culture media and incubated. Any organism which grows must be identified. If it proves to be a saprophyte, this assures the individuals concerned that the room is indeed ready for the next patient. If a pathogen is isolated, further disinfectant procedures are started.

5-87. To offer a means of control and correlation of subsequent information, some standardization is necessary. If you are checking the floor, for example, you should sample a given area each time. To do this, you may make templates or purchase them. You can do air sampling by using an air-sampling centrifuge or by leaving petri dishes containing media exposed to the air for a given period of time. Whichever method you use for sampling, it must be a method which offers a means of standardization and should be used each time.

5-88. If you isolate organisms similar to those with which the patient was infected, you must go over the room and its contents again and recheck sterility. Your responsibility in this area cannot be overemphasized. While collecting the swabs, use initiative and assure yourself that you have obtained a representative cross-sampling of the various accessible areas in the room. Sample the mattress, springs, bed rollers, bedside tables, water carafes, window sills and ledges, floor (center and corners), door ledges, ventilation vents, etc. Only in this way can you check the effectiveness of the disinfecting efforts.

5-89. Of all the problems which confront any hospital, resistant hospital staphylococci is the most frequent. Hospital staphylococci become resistant to many of the efforts used to destroy them. Many hospital personnel carry the organism and transmit it from one part of the hospital to another. The organism can readily invade the meager defenses of the newborn and patients who have their natural defenses lowered because of some other illness.

5-90. *Testing for staphylococci infection.* Staphylococci can be carried from the hospital to the patient's family and friends who, in turn, can become infected. Besides the routine precautions observed in isolating patients with known staphylococcus infections and the complete sterilization of the rooms and equipment with which they come in contact, hospitals are vitally concerned with controlling the transmission of this organism by identifying and treating carriers on its own staff. One of you duties as a laboratory technician will be to culture staff members and determine who might be carrying the staphylococcal organism.

5-91. To culture this organism, you generally use three sites. They are the nose, throat, and lesions. We have already discussed the isolation of organisms from these sources; however, it is pertinent to add here that in order to isolate the staphylococcus organism from the nose, it is not necessary to insert the cotton-tipped swab to the posterior part of the nose. A more realistic approach is to obtain the specimen from the area of the nostril which is exposed to the air. That is, culture the first one-fourth or one-half of the nose. This area has the hairs which filter the air going in and out; therefore, chances of isolating organisms are greater if you culture this area.

5-92. *Testing food and water.* Although primarily a duty of the preventive medicine personnel, an occasion may arise where you may have to examine water, dairy products, and foods for contamination. As these products are consumed by everyone, they must be free of infectious agents. If you should need to examine these poducts, the pocedures are outlined, in AFM 160–52, *Laboratory Procedures in Clinical Bacteriology*.

5-93. **Practicing Aseptic Techniques.** Besides evaluating the aseptic techniques of others, you should be aware of the techniques that you perform routinely. While peforming venipunctures, do you use the correct strength alcohol to disinfect the site of puncture? Are your needles sterile? In bacteriology, do you wipe your benches with a good disinfectant when you finish for the day? Do you wash your hands before leaving the laboratory? Do you exercise the proper care when handling specimens and culture material? Do you perform sterility checks with diligence? These are your individual responsibilities.

5-94. As a laboratory technician, you will come in contact with many patients. Some will have contagious disease and may be in isolation. That is, they are in an area by themselves and so situated that microorganisms stand very little chance of getting out of the area if proper precautions are taken. In another situation, it may be desired to keep microorganisms from entering a patient's room. In both instances, disinfection material will be available to prevent the transfer of organisms from one environment to another.

5-95. In those instances where isolation techniques must be practiced, they must be practiced by everyone to be effective. Although time-consuming, it is necessay to wash your hands in a disinfectant solution, and wear a mask, a cap, a gown, and boots when required. It is also important that you properly dispose of these items. If you must go into a room where there is a contagious disease, take only those materials which you can leave behind. Anything that you remove, such as a tube of blood, must have its external surface decontaminated. Do not take your tray inside. You can very easily contaminate it, and by carrying it through the hospital, contaminate every place you set the tray down. You cannot see microorganisms, so exercise care in everything you do. A good technician does.

CHAPTER 3

Bacterial Morphology and Physiology

TO THIS POINT, you have studied the basic art and science of bacteriology. You should have definite awareness of how to handle and process specimens. Certainly, you should know how to do your work safely, sterilize media and equipment, and use precise aseptic techniques. Without the constant application of this knowledge, your work in the study of microbes is useless, because diagnostic tests in bacteriology usually depend upon isolating the agent of an infectious disease from other organisms present in most clinical specimens and in the laboratory environment.

2. One of the new areas in your studies is the classification of bacteria. All life is divided into two kingdoms, animal and plant. From this point, we will discuss the further breakout in the naming and grouping system until we have finally classified a typical organism.

3. We realize from experience that every living thing has a form and carries out specific functions to maintain its life processes. Bacteria are no different in this respect. You will be able to identify bacteria by knowing their structures, size, shape relationships, and variations from normal appearance and behavior.

4. You need to know what each bacterium looks like, but more importantly you must know how the bacterium likes to live and what it uses for food. You can study its natural environment (in-vivo) and try to duplicate an artificial one (in-vitro) for the bacterium to grow on in the laboratory. This knowledge is very necessary to properly isolate and identify any bacterium.

5. The facts you gather about bacteria can be put together like a puzzle to give their identity. Your "detective attitude" will help you to arrive at an answer. An organism which is fastidious, that is, very exacting in its needs, will concern you more in getting the correct environment for growth. You will have to know a great deal about this basic science to adjust growth conditions to the specific requirements of the "bug."

6. With these areas and problems in mind, in this chapter we will discuss the classification of bacteria, cellular morphology, culturing of bacteria, and the various media that we can use to cultivate bacteria.

6. Classification of Bacteria

6-1. A bacterium should have a name which describes it and conveys a definite idea about the organism. You will work with many different bacteria and use their names in written and oral communication. The names are fairly standard (there are a few exceptions) throughout the scientific field and will convey certain information to your coworkers. In order for you to develop a basic knowledge about the classification and nomenclature of bacteria, we will discuss:

- Taxonomy.
- International method of classification.
- Techniques of classification—morphology and physiology.
- Means of distinguishing species.

6-2. **Taxonomy.** An arrangement of organisms is called a "taxis," so the term "taxonomy" refers to the arrangement and classification of bacteria. Classification systems are somewhat arbitrary and do not directly reflect evolutionary relationships between different groups of bacteria. This fact has made complete understanding and agreement in the naming of bacteria difficult. After all, bacteria lack many attributes enjoyed by other plants and animals. For example, there are no fossils of bacteria to show a distinct line of evolution. Methods of establishing relationships between species on a genetic basis have been found only recently.

6-3. We now know that certain groups of bacteria, as in the higher plants and animals, have a precise sexual mechanism which involves an exchange of cellular material. Moreover, there are a number of other means by which genetic traits from one bacterial strain can be introduced into

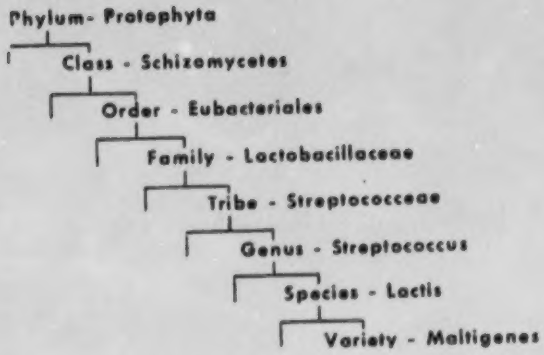


Figure 14. Classification ladder.

closely related organisms. Thus, as progress is made in exploring hereditary mechanisms and relationships, new concepts are being proposed which go far beyond the older ideas of grouping bacteria together on the basis of morphological or cultural similarities. Nevertheless, classification schemes which we will be using for the next several years, still rely largely on the earlier, conventional practices in taxonomy. So these are the schemes on which we will focus our attention for the present.

6-4. **International Methods of Classification.** There are several methods used to classify bacteria. The most common method is based on the *Code of Nomenclature of the Bacteria and Viruses* established by the International Committee on Bacteriological Nomenclature. This Code uses all known properties to classify an organism. These properties include, among others, shape (form), grouping (cell arrangement), motility, Gram-stain reaction, and growth requirements. Our discussion of some of these properties a little later on will show that they fall roughly into three categories: morphology; the physiology of life processes; and a miscellaneous group of characteristics which often serve to differentiate between two organisms that are otherwise indistinguishable.

6-5. A classification system is an arrangement whereby organisms with similar characteristics can be grouped together. As we mentioned previously, all life is divided into either the *plant kingdom* or the *animal kingdom*. Within a kingdom are found very broad and general divisions called *phyla*. Each *phylum* (taken from the Greek word "Phylon" or tribe) contains many different kinds of organisms, but it is represented by living forms which possess one or more similar characteristics. For example, the phylum *Protophyta* contains all the so-called primitive plants, and it is in this phylum that we find bacteria. Thus, the bacteria we are interested in belong to the *plant kingdom* and the *phylum Protophyta*.

6-6. Major differences in organisms allow each phylum to be broken down into smaller groups called *classes*. These in turn are subdivided into *orders*, orders into *families*, families into *tribes*, tribes into *genera*, genera into *species*, and species into *varieties* or *strains*. There may also be *suborders*. Suborders have characteristics distinct enough to differentiate them from the order, but not different enough to warrant their being called families. There are certain endings affixed to some of the taxonomic names. Becoming familiar with these suffixes will help you identify a particular group. The following endings denote groups of organisms at various levels of the classification system:

Name	*Ending*	*Example*
Order	Ales	Pseudomonadales
Suborder	ineae	Rhodobacteriineae
Family	aceae	Thiorhadaceae
Tribe	eae	Eschericheae

6-7. Perhaps we can better understand the system of classification by starting at the bottom and working up the ladder, giving the rules which apply to name an organism. For example, we will look at a single organism, *Streptococcus lactis*, variety *maltigenes*. We normally have only two, rather than three names for organisms. Our example will show how the name is derived in this case where a *variety* is included.

6-8. The first word of a name for an organism is its *genus* name. The first letter is always capitalized. It is usually a Latin or Latinized Greek word, a word derived from the Latin or Greek stem, or occasionally from some other source. In either case, it must be regarded as a Latin noun. Some examples are: *Streptococcus*—chains of a small grain; *Bacillus*—a small rod; *Lactobacillus*—a small milk rod; *Sarcina*—a packet or bundle; *Micrococcus*—a small grain; and *Clostridium*—a small spindle.

6-9. A genus (plural—genera) contains a group of closely related species. There is no agreement as to how many identical characteristics bacteria must have to allow them to be grouped together under a single genus. However, experts on classification have set up a "type species" for each recognized genus. This one species is the yardstick against which unknown organisms are measured with regard to their morphology, behavior in laboratory culture, antigenic makeup, and other properties we have mentioned previously. In fact, comparison with the type species of several closely related genera is sometimes necessary in naming a bacterium, because members of these different genera may share several of the important features which we

TABLE 4
MORPHOLOGICAL CHARACTERISTICS

Shape	Terminology (singular & plural)	Average Size (microns)
spherical	coccus, cocci	1.0
rod-shaped	bacillus, bacilli	0.5 X 2.0
spiral-shaped	spirillum, spirilla	0.2 X 10

(Coccobacillary forms are intermediate between spheres and rods)

commonly search for in the laboratory as a means of identification. The finding of a previously unknown bacterium in a clinical laboratory is a rarity.

6-10. Just as a genus comprises microorganisms which have one or more (usually several) predominant characteristics in common, so a species is a group (within a genus) having certain definite, individual properties that set its members apart from other groups of that genus. The example we chose, *Streptococcus lactis*, is a harmless saprophyte (nonpathogen) normally found in milk. Yet the type species of the genus is *Streptococcus pyogenes*, a pathogen which causes serious infections in man. You can readily distinguish the two species, *lactis* and *pyogenes*, on laboratory media because the pyrogenic streptococcus produces a hemolysin which lyses (ruptures) red cells in blood agar plates. The milk streptococcus is nonhemolytic.

6-11. In looking at the name, *Streptococcus lactis*, we note that the species designation always follows immediately after the genus name. The term denoting the species is an adjective, noun, or verb which is usually descriptive or complementary to the genus. For example, *Streptococcus lactis*—milk streptococcus, *Clostridium Welchii*—Welch's clostridium, and *Clostridium dissolvens*—dissolving clostridium. We see here that the species designation is not usually capitalized, although exceptions can be made if the term was derived from a proper noun, as in *Cl. Welchii*, named for its discoverer.

6-12. A *variety* or *strain* is a subspecies. Organisms of a variety have enough similar qualities to be named after a particular species, but differ enough to be recognized as a group within the species. The variety name follows the species name and is not capitalized, as in our example of *Streptococcus lactis*, variety *maltigenes*. This streptococcus of milk produces a malt flavor. The name can be witten simply as *Streptococcus lactis, maltigenes*.

6-13. Now that we have looked at the bottom of the ladder and have seen how the names of organisms are derived, let's start up the ladder. Figure 14 shows the entire structure of the ladder of classification and how our example fits. A *family* is a group of related genera and/or tribes. Not all families are broken down into tribes and then genera. In some cases, genera follow right below family. The suffix *eae* is added to the stem for tribe name and *aceae* for the family name. In our example, we do have both a tribe and family name. The tribe name is *Streptococceae* and the family name is *Lactobacillaceae*.

6-14. An *order* is a group of related families and is usually designated by adding the suffix *ales;* for example *Eubacteriales*, or the "true bacteria."

6-15. A *class* is a group of related orders. The majority of the organisms which you encounter in the field of medical bacteriology belong to the class *Schizomycetes* ("fission fungi") in the phylum *Protophyta*, the "primative plants."

6-16. As mentioned earlier, there may be other taxonomic categories such as subfamily, tribe, and subtribe. Keep this in mind as you study the microorganisms. It is necessary sometimes for authorities on taxonomy to create a new division for an organism which does not seem to follow the specific classification lines.

6-17. **Characteristics Used for Classification.** We cannot use any one set of characteristics to differentiate all pathogenic bacteria. Classification must be based on many characteristics which we noted before fall into three general categories: morphological characteristics, physiological properties, and other miscellaneous factors which aid in identification.

6-18. *Morphological characteristics.* These include size, shape and arrangement of cells, and internal cellular structures. You can see the three principal shapes of bacteria and their average sizes in table 4. Bacteria vary considerably in size, as the factor of ten times between the average diameter of a coccus and the length of a spiral form shows.

6-19. *Physiological properties.* Classification according to physiological properties is based on growth requirements, primarily the need for an inorganic or an organic source of carbon.

TABLE 5

CELL STRUCTURES

GENERAL

Cell-wall. A thin complex covering of proteins, lipids, and polysaccharides that encloses the protoplasm of the cell, giving rigidity to bacteria.

Cytoplasmic membrane. A semipermeable membrane governing osmotic activity located directly beneath the cell wall.

Cytoplasm. The protoplasmic or vital colloidal material of a cell exclusive of that of the nucleus.

Nucleus. Diffused chromatin material responsible for genetic control of growth and reproduction of the cell.

SPECIAL

Capsule. An accumulation of high molecular weight, excretory substances (slime layer) around a single bacterium or clumps of bacteria. A capsule serves as a defense against phagocytosis Usually the presence of a capsule is a sign of virulence.

Flagellum. A protoplasmic strand of elastic protein originating in the cytoplasm and extending from the body of the cell. A flagellum serves as an organ of locomotion. The arrangement of flagella is characteristic of the species.

Spore. A metabolically inactive resistant body formed by a vegetative bacterium to withstand an unfavorable environment. Only bacilli form spores. The position and size of the spore within a bacillus is peculiar to the species.

Inclusion bodies. Vacuoles of reserve or waste materials contained within the cytoplasm.

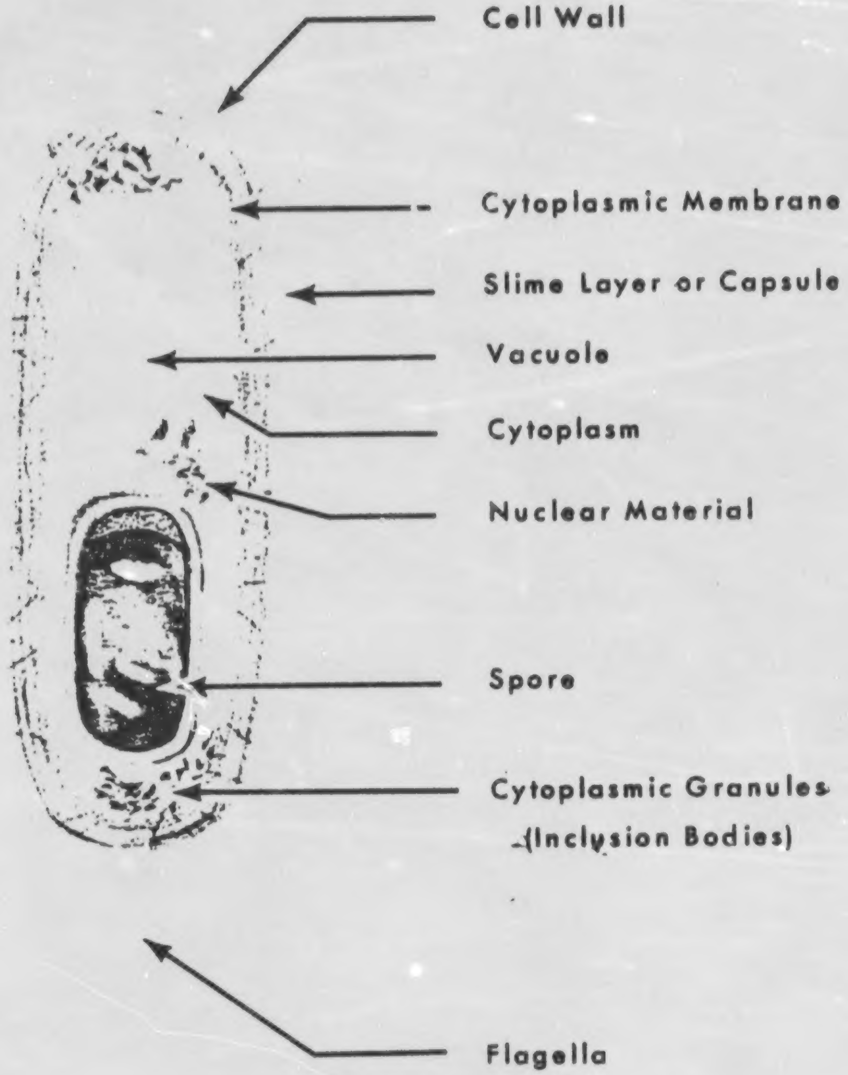

Cell Wall
Cytoplasmic Membrane
Slime Layer or Capsule
Vacuole
Cytoplasm
Nuclear Material
Spore
Cytoplasmic Granules
(Inclusion Bodies)
Flagella

Figure 15. Structure of a Peritrichous bacillus.

6-20. Autotrophic bacteria obtain energy and grow on inorganic media employing carbon dioxide (CO_2) as their sole source of carbon. They begin with CO_2 and ammonia (NH_3) and build an entire protoplasmic structure of protein, fat, and carbohydrates by oxidizing ammonia to obtain energy for their other life processes.

6-21. Heterotrophic bacteria obtain energy from organic carbon sources. Heterotrophs require the addition of sugars, amino acids, purines, pyrimidines, and vitamins to their culture medium. They ferment sugars as their primary source of energy.

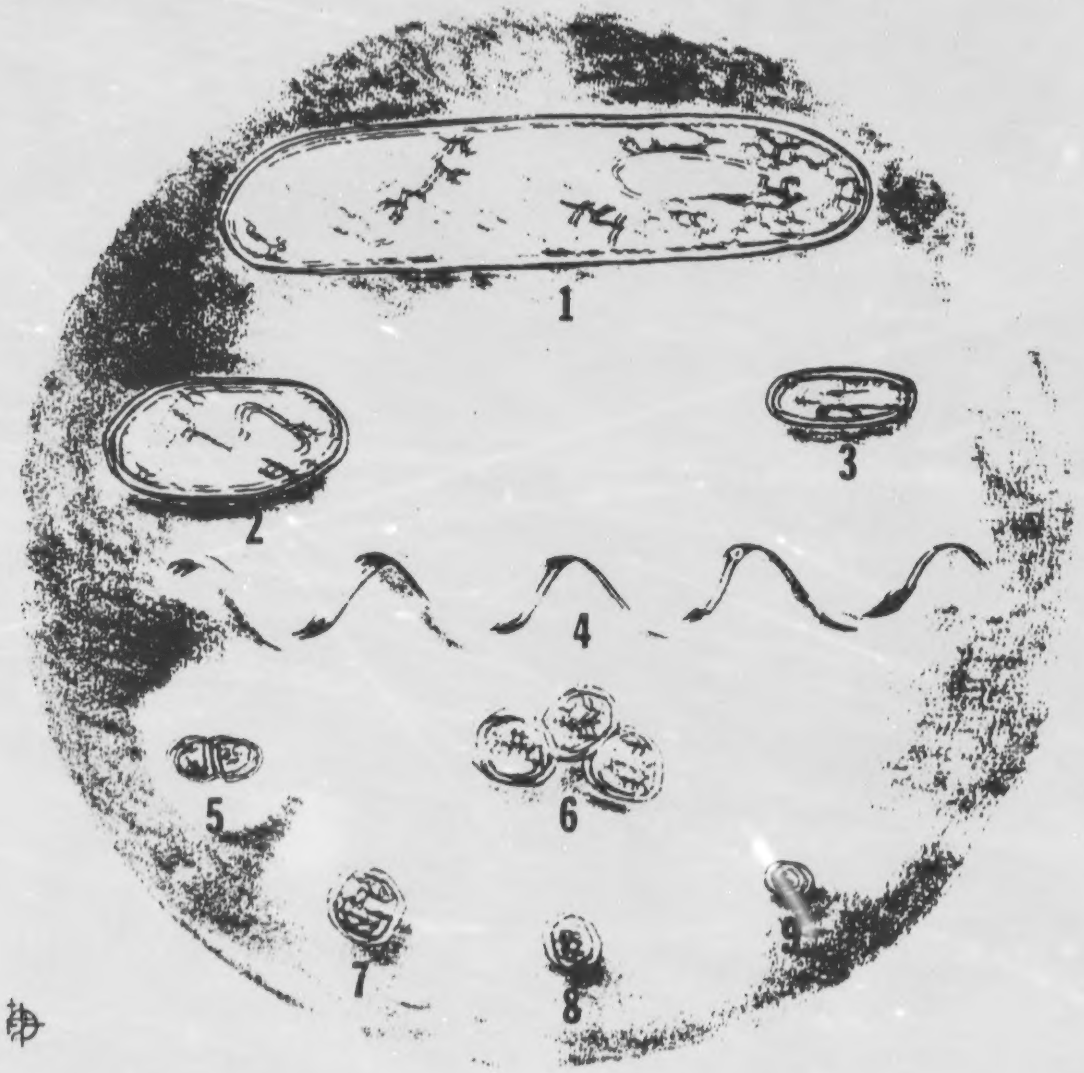
6-22. Disease-producing bacteria are usually heterotrophs. They have adapted to an environment in which many kinds of organic materials are normally available. In many cases such organisms have lost their ability to synthesize from simple compounds certain complex organic substances needed for their growth. Parasitic bacteria (pathogens) require organic matter from a living host for nutrients. Saprophytic bacteria are heterotrophs which use decaying organic matter for nutrients and usually do not cause disease.

6-23. By preparing artificial media containing various nutrients needed for growth and reproduction, bacteriologists are able to study the growth characteristics of bacteria. Growth requirements associated with a parasitic or saprophytic existence in man include optimum temperature, the right concentration of oxygen, proper pH, and suitable nutrients.

7. Cellular Morphology

7-1. Bacteria possess complex cell structures which can be divided into two types: general cell structures possessed by all bacteria and special cell structures possessed in various combinations by some, but not all bacteria. These are defined in table 5 and shown graphically in figure 15. Similarities in cell structure generally make it impossible to classify bacteria on the basis of morphology alone, although the study of size, shape, and structural parts is an important aid to identification.

7-2. **Size of Cells.** In our work we use a unit of measure to compare the size of cells. In the comparison of bacteria to other cells, we use the



1. BACILLUS ANTHRACIS 1 TO 1.3μ BY 3 TO 8.2μ
2. ESCHERICHIA COLI 1 TO 1.3μ BY 2 TO 3.5μ
3. HEMOPHILUS INFLUENZAE 0.3 TO 0.4μ BY 1 TO 1.6μ
4. TREPONEMA PALLIDUM 0.2μ BY 4 TO 15μ
5. DIPLOCOCCUS PNEUMONIAE 0.8 TO 1.30μ BY 1 TO 1.5μ
6. STREPTOCOCCUS SPECIES 0.6μ BY 1.6μ
7. STAPHYLOCOCCUS AUREUS 0.7 TO 0.9μ
8. RICKETTSIA PROWAZEKI 0.3μ BY 0.3 TO 0.5μ
9. VACCINIA VIRUS 0.15μ

Figure 16. Comparison of microorganisms to a red blood cell.

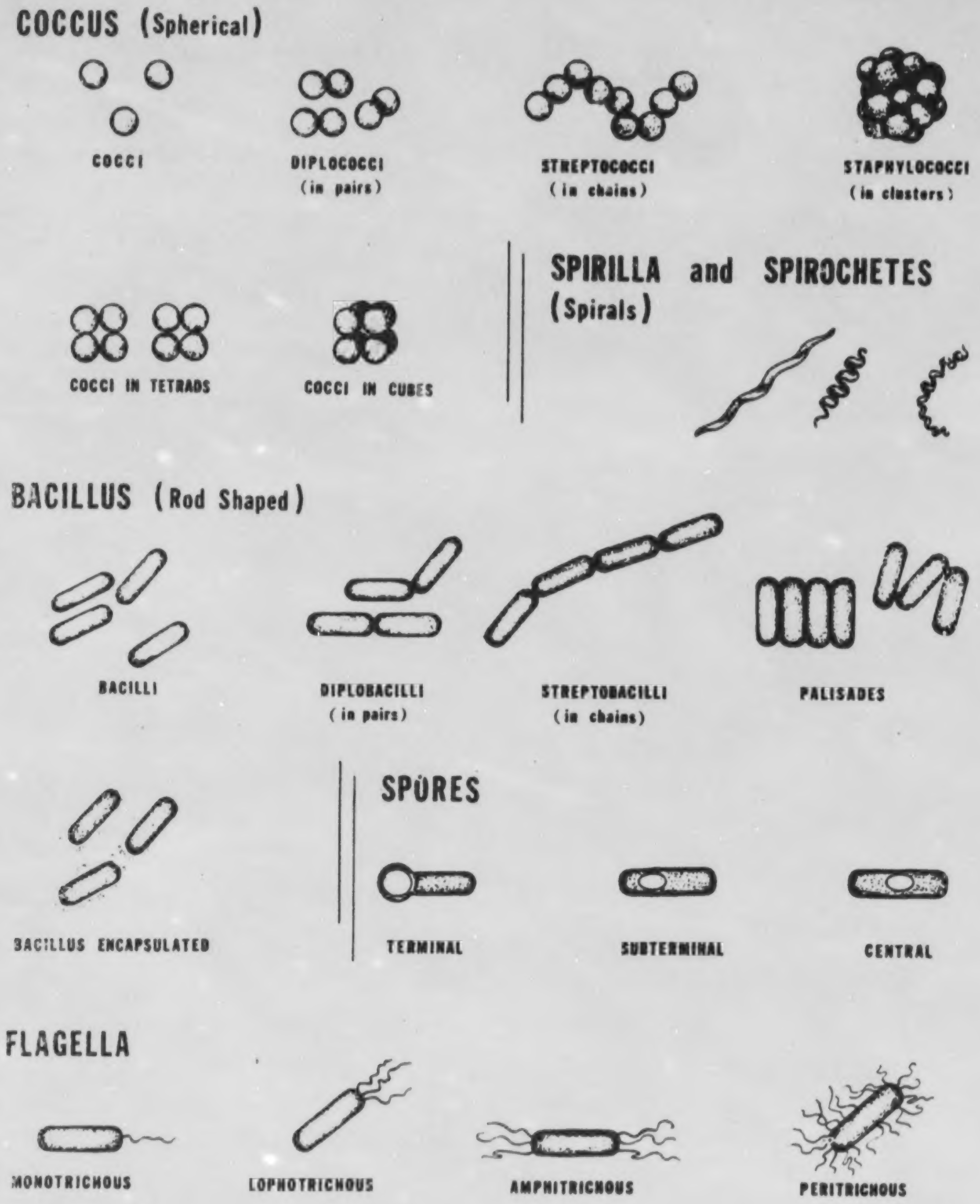

COCCUS (Spherical)
COCCI
DIPLOCOCCI (in pairs)
STREPTOCOCCI (in chains)
STAPHYLOCOCCI (in clusters)
COCCI IN TETRADS
COCCI IN CUBES
SPIRILLA and SPIROCHETES (Spirals)
BACILLUS (Rod Shaped)
BACILLI
DIPLOBACILLI (in pairs)
STREPTOBACILLI (in chains)
PALISADES
BACILLUS ENCAPSULATED
SPORES
TERMINAL
SUBTERMINAL
CENTRAL
FLAGELLA
MONOTRICHOUS
LOPHOTRICHOUS
AMPHITRICHOUS
PERITRICHOUS

Figure 17. Cell structures.

micron (μ). A micron is 1/1000 of a millimeter (mm.) or 1/25400 of an inch. Occasionally, the Angstrom Unit (A.), equivalent to 10^{-7} mm. or 1/10000000 of a mm., is used for very small organisms. Although there are exceptions, most bacteria range from 0.2 microns (width) to 5 microns (length) in dimension. A size relationship can be made using human erythrocytes (red blood cells) which average about 7.5 microns in diameter. We have illustrated in figure 16 a size comparison of microorganisms to a red blood cell. As you become familiar with your microscope, the size of an erythrocyte in the oil immersion field will give you an idea of the dimension of any given organism, and indeed, many measurement references are made in relation to the erythrocyte.

7-3. **Shape of Cells.** As you can see in table 4, there are essentially three different shapes of bacteria. They are spherical, rod-shaped, and spiral-shaped. Figure 17 illustrates the variations in arrangement which these three basic shapes may take. Notice, too, the terminology and position of the bacterial spores and flagella. These descriptive terms are used frequently, and from them you should be able to picture a particular bacterial cell.

7-4. **Grouping of Cells.** Cell grouping as well as the shape can aid in identifying bacteria. The grouping results from the manner in which cell multiplication takes place. Bacteria reproduce by binary fission; by this we mean the bacteria split (divide) at right angles to their long axis. One organism becomes two, two become four, four become eight, and so forth. In the case of binary fission in the rod-shaped bacteria, a cell first increases in length. Then a constriction develops across the narrow width of the cell about midway between the two ends. This is the location at which cleavage (fission) takes place to give us two cells in the place of one. Cell division in the rod forms often gives rise to chains of organisms which break up into single cell masses. However, if individual cells do not separate cleanly, and there is post-fission whipping of bacilli, the side-by-side palisade arrangement shown in figure 17 can result.

TABLE 6

ARRANGEMENT OF BACTERIAL CELLS

Arrangement	Terminology	Type of Division
SPHERICAL FORM		
Single	coccus	--
Pair	diplococcus	in one plane
Chain	streptococcus	in one plane
Cluster	staphylococcus	in many planes
Four	tetrad	two or more planes
Eight	cube	two or more planes
ROD FORM		
Single	bacillus	--
Pair	diplobacillus	in one plane
Chain	streptobacillus	in one plane
Palisade	palisade	in one plane (post-fission whipping)
SPIRILLUM, always occur singly		
COCCOBACILLUS, same as for spherical forms		

7-5. Among the spherical forms (cocci), cell division can occur in more than one plane, and the distinctive grouping of cells which follows reproduction is characteristic of certain genera. To picture this clearly, imagine that we place an orange on a table and slice through the center of the fruit with a knife. The slice can be made from top to bottom (one plane), or laterally from side-to-side (a second plane), or even on the diagonal (a third plane). Also, suppose that each half grows into a whole orange, to be sliced again. A series of oranges sliced each time from top to bottom (only one plane) will yield a chain extending along the surface of the table. This grouping is typical of the streptococci. Similarly, if an orange is sliced first from top to bottom, and the resulting two oranges are sliced in a different plane, the result will be a packet or cluster of fruit rather than a chain.

7-6. In this manner, the grapelike clusters typical of the staphylococci are built up by cell division, first in one plane and then another as reproduction proceeds. In table 6 note that spherical organisms are found in pairs, tetrads (groups of four cells), cubes, and clusters, depending upon the number of planes in which cell division takes place. Incidentally, you can best observe these characteristic groupings in a broth culture rather than in colonies on solid media.

7-7. **Variation.** If we cultivate bacteria under carefully controlled laboratory conditions, we can reasonably expect these organisms to reproduce their typical morphological features from one generation to the next. Likewise, we expect that manifestations of life processes will remain fairly constant from culture to culture of the same organism. But now and then we encounter altered growth patterns in laboratory cultures, or morphological changes that make identification of bacteria difficult. These departures from normal or "typical" growth of a species are called *variation*. Variation is found in form and structure of the cell or colony, metabolic processes, immunological character, and ability to produce disease.

7-8. Variation in bacteria introduces an element of uncertainty which, coupled with the complexity of working with living biological systems, makes the positive identification of microbes one of the most challenging and time-consuming procedures in the clinical laboratory. Fortunately, we ordinarily have sufficient properties that are characteristic of a given genus or species (cellular and colonial morphology, nutritional requirements, immunological components of the cell) to form a composite picture that enables us to establish identity.

7-9. Variation generally arises from either of two causes: a change within the genetic structure of cells in a culture (a mutation); or a noninherited modification in appearance or behavior (an adaptation) brought on by environmental influences as a laboratory culture of organisms grows and reproduces. This second form of change sometimes occurs despite our best efforts to supply a pathogenic bacterium with the same growth conditions it enjoyed in the human host from which it was isolated.

7-10. *Mutations.* Mutations of many kinds occur spontaneously in the genes of the bacterial nucleus. A *gene* is a self-duplicating, biological unit which carries an inherited trait from generation to generation during reproduction. Many mutations are lethal to the organisms in which they occur, but others are not. If environmental conditions favor the growth of a mutant cell, the culture will soon contain a mixture of both typical bacteria and mutants. Resistance to antibiotics is a prime example of a spontaneous mutation among pathogenic bacteria. You can demonstrate this mutation very easily by plating a heavy suspension of organisms to a culture medium containing an antibiotic. A few isolated colonies of the drug-resistant mutant will usually appear, since the inhibitory medium permits survival of only the resistant forms present in the inoculum. The physician must contend with this phenomenon of drug resistance in treating his patient for a bacterial infection. The infectious microorganism, if not already resistant to one or more antibiotics, may develop resistance by mutation during the course of antibiotic therapy.

7-11. *Adaptations.* The second form of variation we spoke of, that is a noninherited modification, or adaptation, of the cell is perhaps best exemplified by changes in size, shape, or structure of bacteria during cultivation in the laboratory. A stained slide prepared from a patient's specimen at the time the specimen is collected for culture will quite often reveal microbes greatly different in morphology from those which grow out on laboratory media. For instance, a cocculike form found in body tissues may emerge as a short rod in the colonies growing on a blood agar plate. Capsules, or a slime layer, are often formed around an organism in the body, only to be lost during cultivation on artificial media. This loss is usually accompanied by a change in colony texture from smooth (S) to rough (R). To cite another example, the bacillus causing the disease anthrax in animals and man tends to remain in the vegetative (nonspore) state during reproduction in the host. Spore formation characteristically takes place only outside of host tissues, or in laboratory media.

53

7-12. There are two other terms which you should become familiar with because they are often used in discussions of microbial morphology and variation—involution forms and pleomorphism. *Involution forms* are abnormal, bizarre, shapes assumed by bacteria in aging cultures, or under highly unfavorable environmental conditions. These forms may bear no clear resemblance to the usual morphological features of the genus to which they belong. For instance, after exposure to antibiotics a short rod may produce, in culture, extremely long filamentous forms because the action of the antibiotic has interfered with the process of cell division.

7-13. Some genera of bacteria are more prone than others to grow in a variety of shapes that can make identification difficult—even in a favorable cultural environment. Species of the genus *Actinomyces*, pathogens of man and animals, are good examples. They have morphological properties akin to those of the molds and higher fungi. Actinomyces are said to be *pleomorphic* in that they occur naturally in several forms: as short coccal forms, bacillary fragments, club-shaped rods, and branched structures which, to the inexperienced bacteriologist, seem to represent a mixed culture.

8. Cultivation of Bacteria

8-1. Any plant or animal will flourish or die, depending on its environment. Given ample food, water, and the proper atmosphere, abundant growth will follow; however, let the opposite be true and death ensues. Bacteria are no different in this regard. They will flourish and reproduce in the laboratory if carefully cultivated.

8-2. You can conclude from our discussion so far that bacteria cannot be identified as to species, and usually not to genus, on the basis of morphology and staining reactions alone. Therefore, you must supplement your microscopic examination by studying the appearance and growth characteristics of bacteria on laboratory media. But in order to set up the proper conditions for microorganisms to survive and multiply on artificial media you must first learn something about microbial growth requirements.

8-3. **Growth Requirements.** To cultivate bacteria in the laboratory you must consider several important factors. These factors are very similar to those involved in the life processes of other plants, animals, and humans. These are:

- Nutrition (proper food).
- Moisture.
- Acidity/alkalinity (pH).
- Temperature.
- Gas Exchange.

8-4. *Nutrition.* The *proper foods* include a source of carbon (organic or inorganic), nitrogen for the production of amino acids and similar products, inorganic chemical salts, and other growth-promoting substances. Most media contain peptone, a breakdown product from protein, which provides an available source of carbon and nitrogen. Certain pathogens need serum or blood in order to reproduce on culture media. Carbohydrates are needed by some organisms as a source of energy, but not by others. You may add calcium, magnesium, potassium, and sodium salts, as well as certain other minerals, and even dyes, to culture media to either assist or inhibit bacterial growth, depending upon your purpose in cultivation.

8-5. *Moisture.* Moisture is essential for growth. There is usually sufficient moisture in a culture medium, particularly in a broth. You can add moisture to closed containers, such as candle jars, by soaking a piece of gauze or cotton with water and placing the dampened material in the container beside the plates of medium.

8-6. *Acidity/alkalinity pH.* The proper *pH* must be maintained during the life of the bacteria. Just as you would die if your body pH changed very much, so will bacteria. Media are usually prepared to a specific pH in the range of 6.8 to 7.2. However, as bacteria grow they utilize the food available to them, and in so doing, change the pH of the medium because of the accumulation of their waste products. To counteract this change, buffering agents are incorporated into culture media. Buffer substances retard changes in the pH of the growing culture, thus, permitting more abundant growth before a limiting acidity or alkalinity is reached.

8-7. *Temperature.* Bacteria vary considerably with respect to their *temperature* requirements for growth. We classify all bacteria into three broad temperature groups: psychrophilic, mesophilic, and thermophilic. Psychrophilic or cold-loving forms grow more readily at lower temperatures, 15° to 26° C.; mesophilic forms grow best at 30° to 37° C.; and thermophilic or heat-loving forms grow best at 50° to 60° C. The majority of human pathogens are mesophilic, growing best at 37° C. A constant temperature incubator adjusted to 37° C. satisfies the temperature requirement for most pathogens.

8-8. *Gas Exchange.* Gas exchange between bacteria and their environment is vital. While most of the bacteria encountered in medical bacteriology will grow under conditions of normal atmospheric oxygen, certain others need more carbon dioxide than is found in air. Still others can obtain their oxygen only from chemicals incorporated in the medium. Since some patho-

60

genic forms can use only one of the several different systems of respiration found among bacteria in general, you must furnish the specific atmospheric environment which those disease agents require. Otherwise, your isolation attempts will almost always result in failure. We will take a closer look at some of the types of respiration found in bacteria, along with other physiological principles important in cultivating microorganisms.

8-9. **Physiology.** Respiration is a process by which bacteria generate energy for growth. You learned in basic chemistry that energy is released through a coupled reaction in which one compound is oxidized while another compound is simultaneously reduced. You will recall that oxidation represents a loss of electrons, and reduction a gain of electrons. In bacterial respiration, electron transport is accompanied by the transfer of hydrogen atoms. So, for all practical purposes the energy-yielding, oxidation-reduction reaction can be pictured as a transport of hydrogen atoms from one compound to another. In other words, an oxidized compound within the cell (hydrogen donor) gives up one or more hydrogen atoms to a second compound (hydrogen acceptor) which is thereby reduced by its acceptance of these same hydrogen atoms.

8-10. Bacteria can utilize a wide assortment of organic and inorganic compounds in generating energy for cell functions. In general terms, if members of a given species can couple hydrogen atoms with atmospheric oxygen (the hydrogen acceptor) as the final link in a chain of oxidation-reduction reactions, then we speak of this process as "aerobic" respiration. If some chemical substance other than free oxygen serves as the hydrogen acceptor, we term this respiration "anaerobic." In bacterial classification, microorganisms can be divided into four major categories on the basis of their requirements for atmospheric oxygen as the hydrogen acceptor:

(1) Aerobes.
(2) Facultative anaerobes.
(3) Obligate anaerobes.
(4) Microaerophils.

8-11. *Aerobes.* An aerobe growing on solid medium must have oxygen gas in the atmosphere surrounding the culture. Broth cultures must contain dissolved oxygen throughout the liquid in order for aerobic respiration to proceed. Quite often the diffusion of oxygen into the broth is accelerated by agitating (shaking) the medium during incubation. In most aerobic organisms the hydrogen atoms arising from oxidation-reduction reactions in the cell are joined to atmospheric oxygen to form hydrogen peroxide (H_2O_2) as the end product in respiration. Since H_2O_2 is toxic to biologic systems, the aerobes produce an enzyme, catalase, to break down the harmful compound into water and oxygen.

8-12. *Facultative anaerobes.* This type of bacterium is able to use either atmospheric oxygen or some other substance as a hydrogen acceptor. By this process, a facultative organism can adapt to grow in either an aerobic or an anaerobic environment. Many of the bacteria which normally live in the human intestine are facultative anaerobes which show rapid growth aerobically on laboratory media. They can adapt to anaerobic conditions when the supply of oxygen in the culture is exhausted. Most of the pathogenic organisms we will study are facultative anaerobes.

8-13. *Obligate anaerobes.* An obligate anaerobe will not grow in a culture exposed to atmospheric oxygen. Free oxygen is toxic to the anaerobe's respiratory mechanism, and hydrogen atoms are usually transferred only between certain organic compounds fabricated by the cell from constitutents of the culture medium. In effect, the anaerobic forms do not possess the respiratory enzymes necessary to couple hydrogen atoms with oxygen, the hydrogen acceptor commonly used by the aerobes and facultative anaerobes. Many of the obligate anaerobes of interest to us in the clinical laboratory, such as the gangrene organisms of the genus *Clostridium*, survive an oxygen atmosphere by forming resistant spores. These spores remain metabolically inactive until an environment free of oxygen stimulates their germination.

8-14. *Microaerophils.* Between the strict aerobes and the obligate anaerobes we find some interesting gradations among bacteria with respect to requirements for atmospheric oxygen. For example, the *microaerophils* are a group of saprophytic (nonpathogens) forms for which oxygen is toxic in normal atmospheric concentration. These bacteria are not true anaerobes because a need for oxygen does exist, although the amount required is extremely small. Then, again, there are so-called indifferent microbes which can grow either in the presence or absence of oxygen. The gas is not toxic to these anaerobic organisms, but atmospheric oxygen is not used as a hydrogen acceptor in respiration.

8-15. **Colony Morphology.** In a suitable environment, a bacterium will grow and multiply. As a result, where originally there was one organism, descendents (progeny) of that organism begin to build up around the original cell. Within a short period of time there are enough organisms in one area to be seen macroscopically.

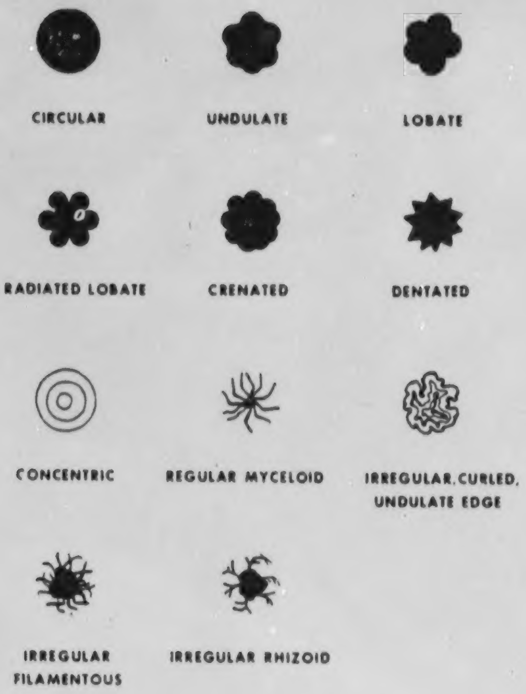


Figure 18. Colony shape.

As these organisms reproduce, they form groups, or colonies, which possess qualities that aid in their identification. The qualities are referred to as colony shape, size, and texture. Recognition of typical colonies of each genus is essential as a step to identification.

8-16. *Colony shape.* Colony shape refers to the outline of the colony, along with its elevation above the medium. When examining colony growth, you must observe individual colonies which have not been distorted or influenced by colonies growing in close proximity. Certain terms are used to describe colonial shape in relation to its outer edge, such as circular, undulate, lobate, crenated, etc. Some colonies may be irregular and appear spread out. You can see examples of colony shape in figure 18.

8-17. The more motile an organism, the greater will be the area of the colony. For example, *Proteus vulgaries* may produce spreading colonies which will cover the entire plate and give the appearance of a thin film of bacteria on the plate—H-type colonies. Proteus may also grow as a small discrete colony—O-type colonies. The H and O designations were derived from the German words, Hauch and Ohne-hauch, denoting spreading and nonspreading forms. The H-type colony is due to active flagellar motion, and the O-type colony indicates a nonflagellated and, therefore, nonmotile organism. It is significant that the presence of flagella correlates with the antigenic properties of the organism. *Antigens* are chemical components of the bacterial cell which stimulate the formation of specific protective substances, "antibodies," in the bloodstream of man and animals. You will learn later how to capitalize on this phenomenon by using serological techniques to detect differences in antigenic structure of closely related microorganism. Sometimes you can distinguish morphologically identical species only by means of analysis of their antigens.

8-18. Colony elevations are described by such terms as flat, convex, umbilicate, raised, umbonate, papillary, and convex rugose, as we have illustrated in figure 19. To best view these characteristics, you should use oblique light and a hand lens to see shadow effect. Older colonies may tend to flatten and yield nontypical features. Be aware of the time element.

8-19. *Colony size.* The size of a colony is labeled as dwarf (diphtheroidal), pinpoint, small, discrete, large, or spreading. Under normal cultural conditions, size is an aid in identification. Significance is often attached to minute (dwarf) colonies in that these are apt to be variant colonies having a degree of virulence different from that of larger colonies of the same organism. As with the shape of the colony, size can be affected by factors of motility. Distortion in size may also be caused by the presence of too much moisture on the medium surface. This is one reason why you should not inoculate media surfaces with bacteria while water droplets, arising from condensation, are still present on the culture plate. The resulting growth may not take the form of isolated colonies needed as an aid in identification.

8-20. You can gather other information from tube cultures. In figure 20, you can see the different growth patterns of organisms on a slant culture. For certain identification procedures, you may do a stab culture to examine the growth of organisms within a solid medium. Figure 21 shows the growth patterns in a solid medium. The pattern obtained aids in identification. We will discuss the preparation of these cultures in Chapter 4.

8-21. *Colony texture.* Another useful aid to identification is the texture of the colony. Texture is described by such terms as rough, smooth, glistening, mucoid, heaped, etc. Some colonies are so mucoid that, if you touch them with a metal loop, you will see a long string of sticky material adhering to the loop as you withdraw it. Mucoid colonies can result from heavy capsule formation by individual cells, or by secretions produced by bacteria in response to an external stimulus, such as phenol in a medium.

8-22. Smooth (S) and rough (R) colonies are frequently encountered. Many bacteria produce both the S- and R-type colonies. The S-type colony is generally smooth, translucent, convex, glistening (moist), circular, and "butterlike" in consistency. The S-type bacteria are usually encapsulated and, as a rule, will produce a smooth growth in a liquid medium or broth.

8-23. Loss of virulence or capsule may cause the bacteria to show a variation in colonial growth: that is, instead of an S colony, an R colony is produced. An R colony is generally dull in appearance, less translucent (more opaque) than the S colony, and may have an irregular circumference and a wrinkled or rough surface. Bacteria from R colonies, when grown in broth, may form flakes or granules. They may settle to the bottom or form a surface scum or pellicle. Stained slides made from the broth, or from surface colonies from the plated medium, will usually reveal long, filamentous organisms.

8-24. **Bacterial Pigments.** In addition to shape, size, and texture, bacterial colonies can develop certain characteristic colors or pigmentation, or cause the surrounding medium to become pigmented. Although we don't know exactly how these pigments are formed, they often give us a hint as to the identity of organisms growing on plated media or in a culture tube. Colors are produced in a wide range, depending upon the type of medium used, time and temperature of incubation, age of culture, and other environmental factors. On a dye-free medium the colonies of most bacteria are white to gray and range from opaque to translucent. But with the pigmented media, a colony may be a bright yellow on one medium, and a dull yellow, orange, or white on another.

8-25. The two major groups of pigment that you may encounter are *leukobases* and *lipochromes*. Leukobases are compounds which, after being excreted by the bacteria, are oxidized to form a colored product. An example of this reaction is found in *Pseudomonas aeruginosa*.

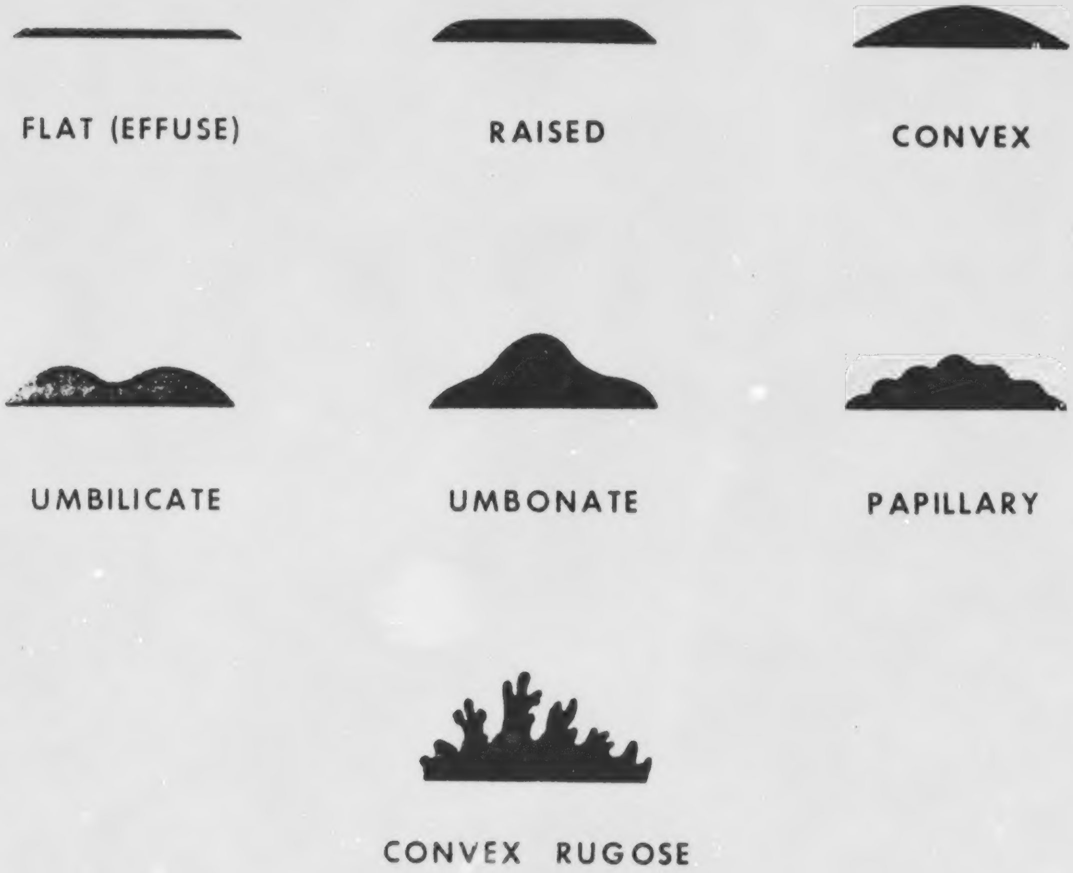

FLAT (EFFUSE)
RAISED
CONVEX
UMBILICATE
UMBONATE
PAPILLARY
CONVEX RUGOSE

Figure 19. Colony elevations.

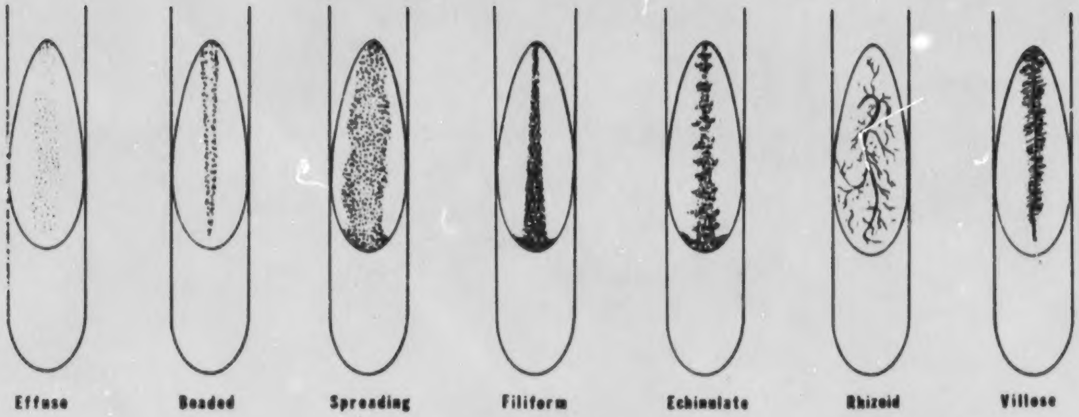


Figure 20. Slant streak culture.

The organism produces a blue pigment, pyocyanin, which gives us the term "blue pus" that is descriptive of certain wound infections. Lipochrome pigments are red, yellow, and orange; they are similar to those produced by flowers and to pigments seen in butter and egg yolk.

8-26. Pigments vary a great deal in color intensity, even with the same organism. Moreover, they are unstable substances. Only if you interpret pigmentation with certain considerations in mind (i.e., the conditions of cultivation,) can pigment formation be used as an identifying characteristic. There are three types of pigments of interest to you, based on solubility differences in common laboratory solvents. They are (1) carotenoids, (2) anthocyanins, and (3) melanins. In table 7, we have listed the types of pigments, their colors, and some of the organisms which produce them. These bacterial genera are often found as isolates from clinical specimens.

8-27. **Anaerobic Cultures.** In our earlier discussion of anaerobic organisms, we mentioned decreased oxygen tension as a requirement for growth. The Difco manual[2] lists the following means of obtaining partial or complete anaerobiosis:

a. Addition of small amounts of agar to liquid media.
b. Addition of fresh tissue to the medium.
c. Culturing in the presence of aerobic organisms.
d. Addition of a reducing substance to the medium.
e. Displacement of the air by carbon dioxide.
f. Absorption of the oxygen by chemicals.
g. Removal of oxygen by direct oxidation.
h. Incubation in the presence of germinating grain or potato.

---

[2] *Difco Manual*, 9th edition, Difco Laboratories, Detroit, Mich.

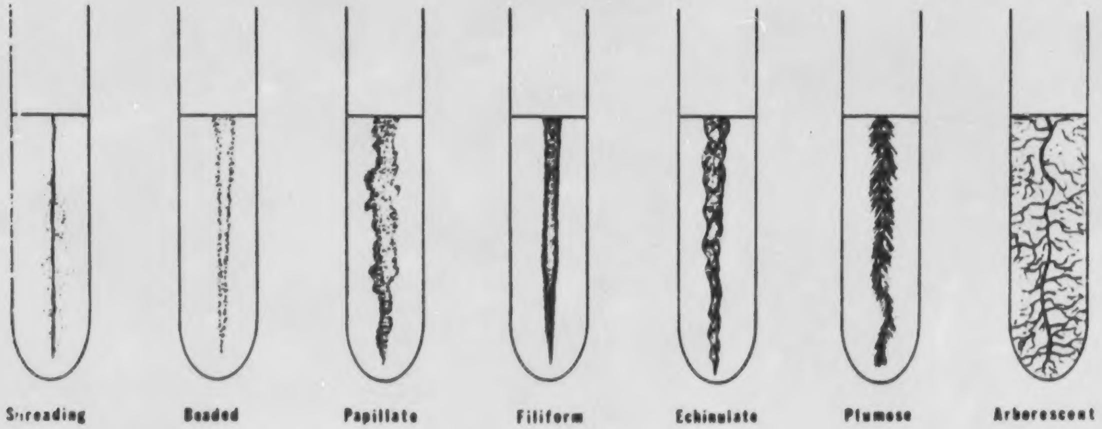


Figure 21. Tube stab culture.

TABLE 7

BACTERIAL PIGMENTS

Type Pigment	Color	Representative Genera
Carotenoid	Red, yellow, orange	Sarcina Micrococcus Staphylococcus
Anthocyanins	Blue, red, intermediate colors	Pseudomonas
Melanins	Red, brown, orange, black	Bacillus

i. Inoculation into the deeper layers of solid media, or under a layer of oil in liquid media.

j. Combinations of these methods.

As you can see, there are numerous techniques to accomplish the various degrees and special states of anaerobiosis. However, in daily practice relatively simple procedures are available and in common use.

8-28. *Thioglycollate broth.* This is a medium which contains a small amount of agar in a liquid medium. It is excellent for cultivating strict anaerobes as well as aerobes. The sodium thioglycollate in the medium acts as a reducing agent and complete anaerobiosis is produced in all but the very upper portion of the medium. Thioglycollate fluid medium is widely used as a routine broth medium for most bacteriological analyses.

8-29. *Burning candle method.* Many microorganisms require a carbon dioxide concentration above normal atmospheric levels. You can supply an atmosphere containing 2 to 3 percent carbon dioxide by incubating cultures in a candle jar as shown in figure 22. Any wide-mouth vessel with an airtight lid will suffice, provided it is large enough to accommodate several cultures. Place the inoculated tubes or plates of media in the vessel, then place a lighted candle in the container above the cultures, and securely tighten the lid. The burning candle will be extinguished when about 2 to 3 percent carbon dioxide has accumulated. Be certain to place the candle as high as possible in the candle jar to permit accumulation of CO_2 which extinguishes the flame. (The CO_2 is heavier than air and fills the bottom first.)

8-30. ***Brewer jar.*** The Brewer anaerobic jar shown in figure 23 is a large, cylindrical vessel which will accommodate several agar plates and test tube cultures. Either hydrogen or natural gas may be used to create the desired atmosphere. Since natural gas is readily available, it is far more practical and less dangerous to use in securing anaerobiosis with a Brewer jar.

8-31. When operating a Brewer jar with natural gas, place a tube of soda lime inside the

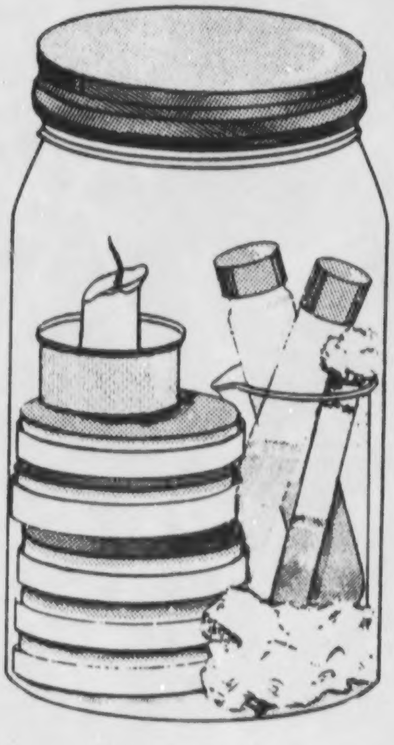


Figure 22. Candle jar.

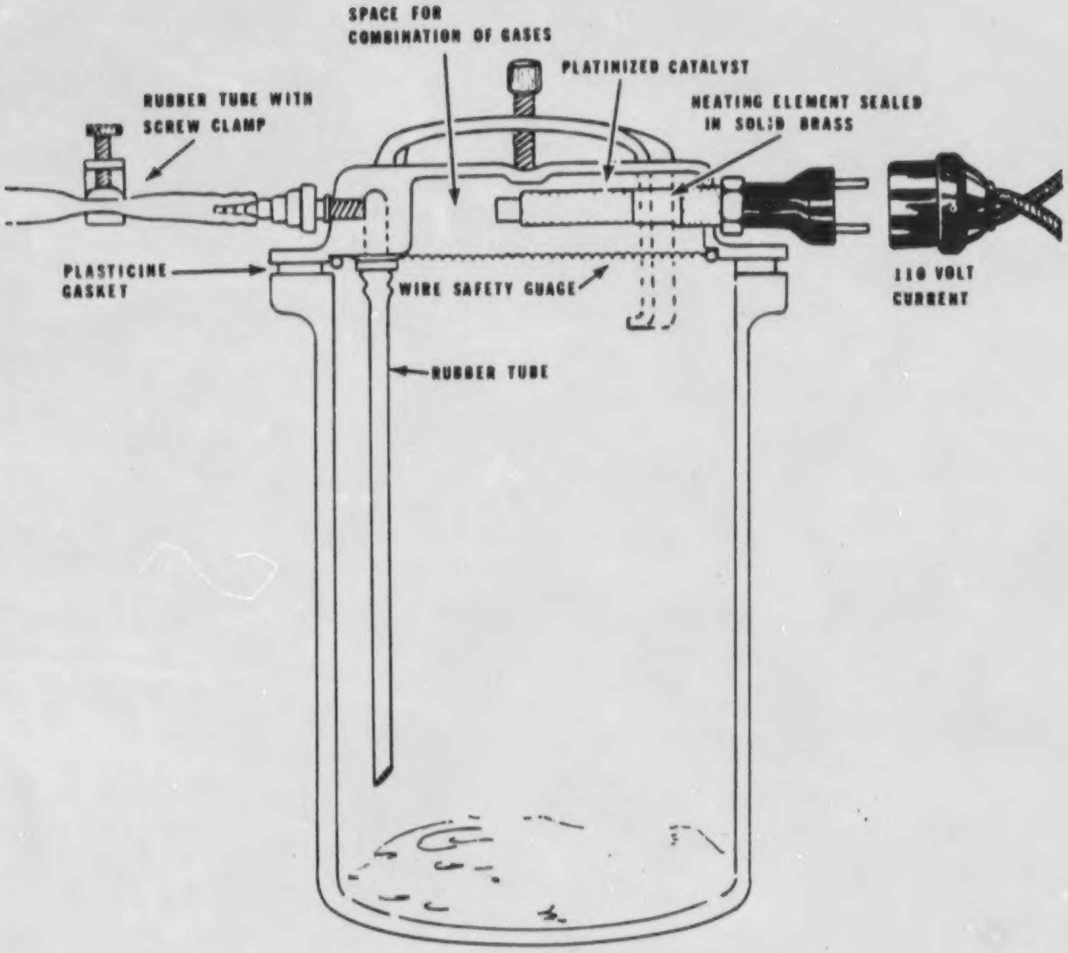

SPACE FOR COMBINATION OF GASES
PLATINIZED CATALYST
RUBBER TUBE WITH SCREW CLAMP
HEATING ELEMENT SEALED IN SOLID BRASS
PLASTICINE GASKET
WIRE SAFETY GUAGE
110 VOLT CURRENT
RUBBER TUBE

Figure 23. Brewer anaerobic jar.

jar along with the cultures. The soda lime will absorb the excess carbon dioxide formed and, thereby, prevent a lowering of pH in the culture media. During the process of obtaining anaerobiosis, proper functioning of the apparatus is shown by the formation of droplets of water on the inside of the jar.

8-32. *Pyrogallol technique*. Another anaerobic method is the pyrogallol anaerobic technique. Pyrogallic acid and sodium carbonate are mixed in an airtight system, the oxygen is absorbed by alkaline pyrogallol, and an anaerobic atmosphere prevails. This technique can be modified for any leakproof vessel which will accommodate cultures. Standard supply items are available for this technique also.

8-33. *Bray dish*. The Bray dish used for this technique is illustrated in figure 24. One side of the divided Bray dish bottom is charged with a 40 percent solution of pyrogallic acid. A 20 percent solution of sodium carbonate is added to the other side of the Bray dish bottom. You should take care to prevent mixing of the solutions at this stage. Invert the inoculated agar dish and place it over the wide mouth of the Bray dish. You should carefully seal it to the Bray dish with a strip of parafilm, or suitable sealing material. Gently rock the vessel, thoroughly mixing the pyrogallic acid and sodium carbonate solutions. This will cause absorption of the oxygen and create an anaerobic atmosphere.

8-34. *Sodium carbonate method*. A modification of the Bray dish technique is to use a section of filter paper fitted inside the lid of a Petri dish, as shown in figure 25. A pinch (approximately 0.5 g.) of crystalline pyrogallic acid and a pinch of crystalline sodium carbonate are mixed together on the filter paper. Moisture for the reaction will be obtained from the agar.

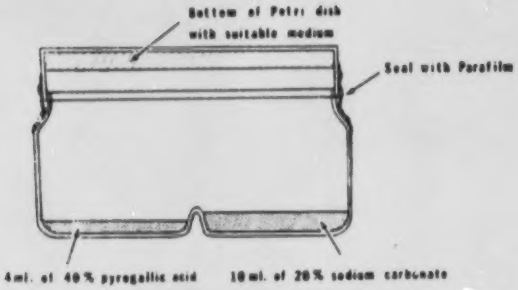


Figure 24. Technique for bray dish.

Place the inoculated agar dish on the lid and carefully seal it with suitable sealing material.

8-35. You must incubate cultures in the usual inverted position so that the reagents or filter paper will not contaminate the culture. When several tubes or plates are to be incubated anaerobically in a large, leakproof vessel, the usual proportions of reagents are 10 g. of pyrogallic acid and 100 ml. of 20 percent sodium carbonate per liter of air space.

8-36. To obtain an atmosphere of 10 percent carbon dioxide, you can use another method. Invert the inoculated medium plate and place it in a vessel equipped with an airtight lid. Place one gram of sodium carbonate for each liter of vessel capacity in a small open container inside the vessel. Cover the sodium carbonate with cotton and add 100 ml. of 3 percent sulfuric acid for each gram of sodium carbonate used. Then, securely fasten the lid.

9. Culture Media and Environment

9-1. Bacteria had been grown in culture broths for some time before Robert Koch, the German physician, began experimenting with solid media in the 1800s. He first used 2 to 5 percent gelatin, which worked well in cool temperatures or when the bacteria did not hydrolyze the gelation. Frau Hesse, wife of one of his investigators suggested agar-agar, a seaweed preparation commonly called agar. Agar had come to European kitchens from Java and was used in preparing jellies. This new material seemed inert to the action of bacteria. It launched a whole new era in the study and isolation of bacteria.

9-2. Since Robert Koch's introduction of solid culture media for growing and isolating bacteria, the field of bacteriology has expanded and prospered. A great deal of research has been done to prepare an artificial medium which is similar to the environment each organism prefers in nature. In order for bacteria to build up their cells and reproduce, the elements necessary for the various cell structures and functions must be supplied in the immediate environment.

9-3. **General Classes of Media.** No single culture medium is satisfactory for cultivating all bacteria. A general purpose or basal culture medium has sources of carbon, nitrogen, and inorganic salts; but the addition of other substances, such as blood, serum, amino acids, or vitamins may be required for growth of pathogenic organisms. The basic energy sources are supplied by water extracts or infusions of meat, vegetables, or yeast. Other complex energy sources include hydrolysates or digests of protein which yield the nitrogen-containing amides, amino acids, peptides, peptones, and proteoses.

9-4. Additional energy sources may be supplied by carbohydrates, alcohols, organic acids, glucosides, or similar materials. Carbohydrates, especially glucose, are often incorporated into media for the primary isolation of pathogens. When studies for hemolysis are to be performed, however, carbohydrates should be excluded from blood agar for they tend to inhibit the bacterial production of certain hemolysins.

9-5. Culture media may be liquid or solid. You can prepare a solid medium by adding agar to a liquid nutrient medium. As previously mentioned, agar is an inert, purified extract of *Gelidium* species, a type of seaweed, which is not metabolized by bacteria. Heat a liquid medium with added agar powder or flakes to melt the agar and then sterilize it. At a temperature of 96° to 98° C. agar melts and goes into solution. Upon cooling to 40° C., the agar solidifies and congeals the medium. You generally use agar in 1.5 to 2.0 percent concentrations in preparing solid media. Lower concentrations give a semi-solid medium which is ideal for performing motility checks of pure cultures.

9-6. Your main purpose in culturing bacteria is to produce pure cultures on a solid medium. Therefore, a very important factor in media preparation is sterility. Media must be free of bacteria before inoculation so that growth of the organism from a clinical specimen will not be masked or inhibited by contaminants. The most common means of sterilizing media is by the auto-

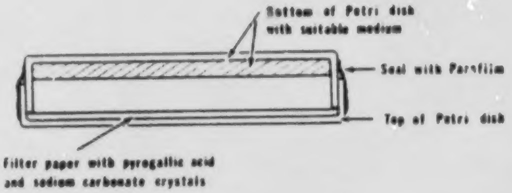


Figure 25. Technique for petri dish.

clave in which steam under pressure is used to reach the necessarily high temperature of about 121° C.

9-7. Prolonged heating at high temperatures can change the composition of a medium. For example, excessive heat can chemically break down lactose broth, and may demonstrate a positive lactose fermentation (false positive) by organisms which do not ferment lactose. Increased heat may also make a medium more acid than desired, or form an undesirable precipitate which will be visible in the medium. The gelling ability of the medium may also be destroyed.

9-8. When you prepare media from commercial sources, follow the manufacturer's direction very carefully as to time and temperature of ster-

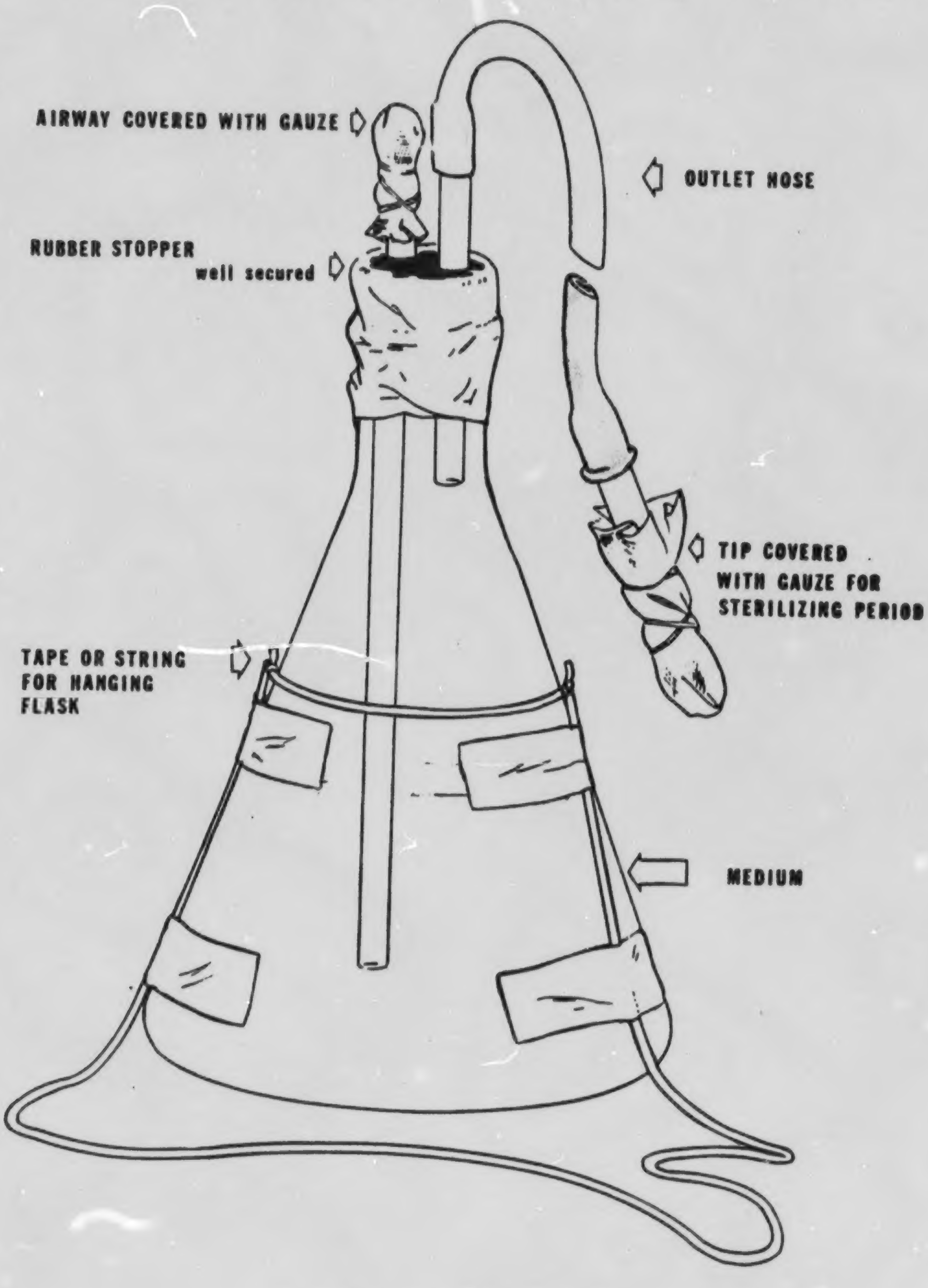

AIRWAY COVERED WITH GAUZE
OUTLET HOSE
RUBBER STOPPER well secured
TIP COVERED WITH GAUZE FOR STERILIZING PERIOD
TAPE OR STRING FOR HANGING FLASK
MEDIUM

Figure 26. Flask of sterilized medium.

ilization. Much of the media used today is commercially prepared and available in powdered, dehydrated form. This powder contains the ingredients necessary to grow the bacteria for which it was specifically designed and needs only to be reconstituted with a fluid and sterilized before use.

9-9. In order to select and prepare cultures for various bacteriological analyses, it is necessary for you to become familiar with the many and varied types of media. We can break the media we commonly use into the following principal types:

- Basal.
- Enriched.
- Isolation (differential and selective).
- Biochemical.

9-10. *Basal.* Basal media contain ordinary peptone, peptone and meat extract, or peptone and meat infusion, with 0.5 percent sodium chloride. Agar is added when solid media are required. Such basal media are used for growing nonfastidious organisms which comprise the majority of bacteria.

9-11. *Enriched.* Enriched media are usually prepared by adding blood, serum, ascitic fluid, amino acids, or other complex nutrients to a basal medium for the primary isolation and subculture of fastidious organisms (usually pathogens). The sterile enrichment substances must be added *after* sterilization of the basal media.

9-12. *Isolation.* Isolation media are used to obtain pure cultures of pathogenic microorganisms from a heavily contaminated source such as a fecal specimen. Specific agents are added to the media to retard or prevent the growth of contaminants present in the specimen without affecting the growth of any pathogens. When you use such media in the solid state, you may add specific carbohydrates and acid-base indicators to give gross differentiation between organisms on the basis of fermentation reactions. Isolation media are subdivided into differential and selective media.

9-13. *Differential.* These media are those that contain chemicals which allow the differentiation of bacteria according to growth characteristics. For example, a certain medium contains the carbohydrate lactose and an indicator (phenol red). We use this medium to help us differentiate between lactose and nonlactose fermenters. If the lactose is fermented, an acid byproduct is produced which will give a visible color change in the medium.

9-14. *Selective.* These media are those which allow only specific organisms to grow. Chemicals are incorporated into the medium which allow the growth of select organisms. An example of this type of medium is *Salmonella-Shigella* (SS) *Agar.* This medium allows few organisms other than *Salmonella* and *Shigella* species to grow. In some cases, a selective medium can distinguish between genera. SS medium will grow *Salmonella* and *Shigella* species. But *Brilliant Green Agar* will grow *Salmonella* and inhibit *Shigella.*

9-15. *Biochemical media.* Biochemical media are employed to detect the enzyme activity of bacteria. The ability to hydrolyze urea to ammonia and carbon dioxide, to ferment various carbohydrates to produce identifiable acids and gas, to reduce nitrates to nitrites, or to produce indole from tryptophane are all examples of enzymatic activities which are useful in differentiating bacteria. Any medium which will permit the detection of these or comparable reactions may be termed a biochemical medium.

9-16. **Preparation of Media.** The proper preparation of a medium is as important as its selection. We should reemphasize here that in preparing any medium from commercially manufactured ingredients, you should always follow the manufacturer's directions explicitly. As stated previously, most of the media you will use are purchased as dehydrated powder from commercial laboratories.

9-17. *Rehydrating media.* In rehydrating the media, you should use distilled water. Fresh, distilled water is best because water which has been stored in an open container for long periods of time can absorb enough gases to alter the final pH of the medium. Carefully weigh the dehydrated powder on weighing paper according to the directions on the container label. Slick paper is preferred over filter paper when weighing, as some of the powder may be lost by adhering to the filter paper. You should then place the powder in a dry flask of a size suitable to hold twice the final volume of medium desired. For example, 1 liter of broth should be prepared in a 2-liter flask. You should add water to the dehydrated medium slowly at first to make sure that it will be completely dissolved. Add a little water and make a thick slurry. Then, you can add the remaining water. If you dissolve dehydrated media in this way, there will be no lumps and you will prevent burned agar when heat is applied.

9-18. In preparing an agar-type medium, you must apply heat and bring the solution to the boiling point. Agitate and swirl the flask to prevent burning and sticking of the medium to the flask. Use caution while heating solutions over the burner as the medium may boil over the top and onto your hands, causing serious burns. Watch the flask carefully and hold it with tongs or asbestos gloves. Keep the opening of the flask pointed away from your face. If you notice that the material is about to boil over, set the flask

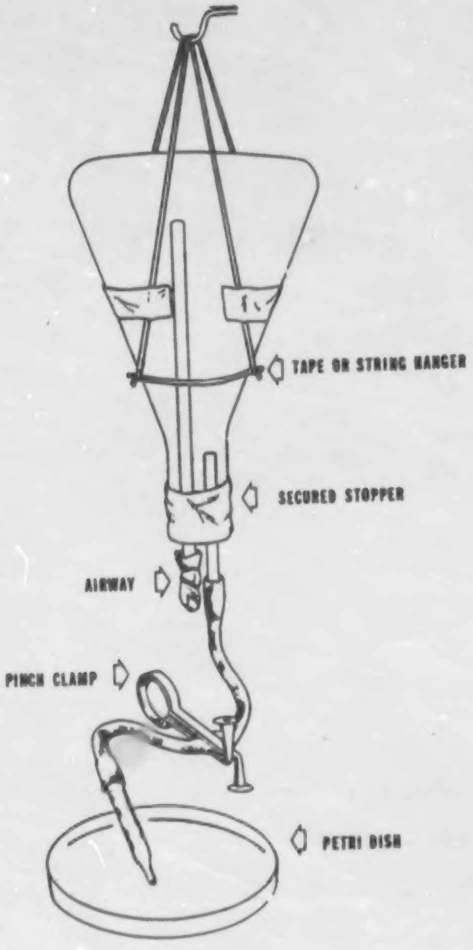


Figure 27. Flask setup for dispensing media.

down immediately. Do not attempt to carry the flask to the sink. You can clean up a mess and make more media a lot easier than you can treat burned hands and fingers.

9-19. *Using Liquid Media.* You do not need to heat most liquid media to boiling because they are usually soluble in water at room temperature. You can best dissolve a medium containing gelatin by heating it to a temperature of 50° C. in a water bath.

9-20. We cannot overemphasize the importance of exercising extreme care in media preparation and also the necessity of using chemically clean glassware. Detergents or other contaminating chemicals may act as disinfectants or alter the pH of a medium. The medium will not have the proper environment for the bacteria we are trying to identify.

9-21. *Quantity of media.* The larger the volume of material to be sterilized, the longer it takes to sterilize completely. As a general rule, however, the quantities of media you will be preparing in the laboratory can be sterilized in 20 to 30 minutes at 121° C. During autoclaving there is considerable bubbling. Allow extra space in the flask for this bubbling effect to prevent overflow and loss of medium. As the medium begins to boil during heating, it tends to expand. An Erlenmeyer flask is tapered toward the top, so this constriction speeds up the rate at which the medium can bubble over. Using a larger flask will eliminate this problem, as we noted in the example of a 2-liter flask to hold 1 liter of medium.

9-22. *Filtering media.* When you sterilize media by filtration because some of the ingredients would be destroyed at autoclave temperature, you must dissolve the ingredients slowly over low heat to prevent the breakdown of constituents and formation of lumps which would clog the filter. It is important that all equipment and tubes be sterile when using filtration and that you use aseptic technique to maintain sterility of the filtrate.

9-23. *Enriching media.* When you need to add enrichment materials to a medium, you must cool the medium to a temperature which will not alter or destroy the additives. As a rule, after the medium has been autoclaved, it should be cooled to 45 to 55° C. before adding substances such as whole blood, ascitic fluid, albumin, or any material which can coagulate or be destroyed by high temperatures.

9-24. *pHing media.* It is not usually necessary to adjust the pH of a sterilized medium when using the commercially prepared powder, provided that you have carefully followed the manufacturer's directions. The final pH will be as stated on the label. When you are preparing your own medium from a formula and stock chemicals, however, you should check the pH with a pH meter and add a small amount of a weak base or acid as indicated by whether the medium is too acidic or too alkaline, respectively.

9-25. To use media for cultures, you must have them in small, usable portions or aliquots. These aliquots are dispensed into sterile containers suitable to whatever purpose that is needed. The containers may be Petri dishes, test tubes, bottles, or glassware of some special design. Most important, you must dispense the culture material into the sterile containers (glass or plastic) in such a way as to prevent contamination of the medium, bubbles on the medium surface, and lumps in the medium.

9-26. *Preventing contamination.* To prevent contamination of the medium, be certain that the bench area is disinfected. It should be wiped clean with any of several disinfectants kept in the lab-

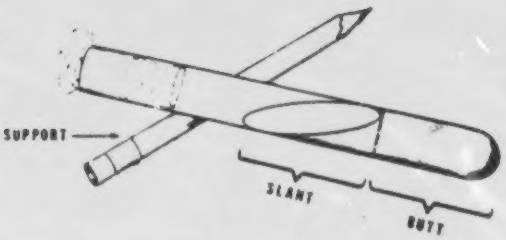


Figure 28. Diagram of typical slant culture.

oratory for this purpose. Leave the petri dishes covered until you are ready to pour the medium, then remove the tops only long enough to add the medium. Pouring should take place in an area free of air currents and away from other laboratory activities which might cause dust in the air.

9-27. *Preventing bubbles.* Bubbles on the surface of the medium can be dangerous to you. They also create a problem in trying to obtain isolated colonies. Bubbles will catch and "spring" a wire loop as it passes over the medium. This spring or snapping effect may cause an aerosol, that is, an airborne cloud of particles or droplets containing infectious bacteria. It is also possible for the wire loop to cut into the medium around the bubble and in so doing be wiped free of most bacteria. Very few bacteria will be left on the loop to streak out for isolation as individual colonies.

9-28. To prevent bubbles in the medium, you can use a sterile gauze over the opening of the pouring flask. As you pour the medium through the gauze, the bubbles are blocked and broken up. When pouring, it helps to have the mouth of the flask close to the petri dish to avoid splashing and aeration bubbles.

9-29. Another good method to prevent bubbles from accumulating on the medium surface, prevent contamination, and make distribution of the aliquots easier is to use an Erlenmeyer flask setup, as shown in figure 26. This device allows for a one-man operation, and more media can be poured at any given time because many petri dishes can be placed in position near the operator. You can sterilize the flask and its contents as an intact unit before pouring the medium. In figure 27, we show the flask hung for dispensing medium to containers.

9-30. The most common method to break up bubbles on an agar medium surface is to gently pass a Bunsen burner flame over the medium before it hardens to burst the bubbles and allow the medium surface to smooth out. Take care, however, not to melt plastic petri dishes with the flame. If the plastic bottom lip gets soft and you lower the top, the two parts may adhere to one another making it difficult to separate them later on.

9-31. *Preventing lumps.* Lumpy medium results from allowing the medium to cool too much before pouring, that is, to the point at which the agar begins to solidify. A lumpy medium, although suitable for bacterial growth, does present some of the hazards described for a medium which bubbles on its surface. A lumpy medium does not usually have an even distribution of nutrients. This gives problems when you try to interpret the results of your isolation technique, because the same organism may give a different colony appearance on various parts of the plate, and it is difficult to streak the plate. Do not allow the medium to cool to the point of solidification before pouring your aliquots.

9-32. The use of test tubes to hold media is universal. You can use test tubes to hold liquid, semisolid, and solid media. You may allow solid media to solidify in the tube standing upright or tilt the tube before hardening to give a solid medium with a slanted surface. All tests tubes must be sterile and chemically clean. They must be stoppered with cotton, gauze plugs, or plastic plugs or covered with metal lids. You may also use screw-capped tubes. Choose the tubes according to the type of medium being prepared and the purpose of its use. Thrift should not be the main reason for using small test tubes to dispense media; however, use the smallest tube which will serve the purpose. Some media-making instructions list the size tube in which the medium should be dispensed to obtain best results.

9-33. ***Preparing slants.*** **To prepare slants, dispense the heated medium into test tubes and autoclave it. After that, tilt the tubes by some means to offer the proper slant area. Once slanted, do not disturb the tubes until the medium is completely gelled. In figure 28, we have shown a correctly slanted tube. There should be an adequate butt and slant area if this form of medium is to be technically useful. The slant is commonly used in the identification of enteric organisms. It provides both an aerobic condition on the surface of the slant and anaerobic conditions in the butt. Depending on the specific medium used, the size of the butt can vary. A butt one-half the length of the slant is usually satisfacory.**

9-34. *Dispensing media.* You can dispense a medium into test tubes by several methods. Use a funnel, rubber tube, and pinch clamp setup to dispense approximate amounts in each tube. The best technique is to use an automatic syringe as shown in figure 29 that measures the desired vol-

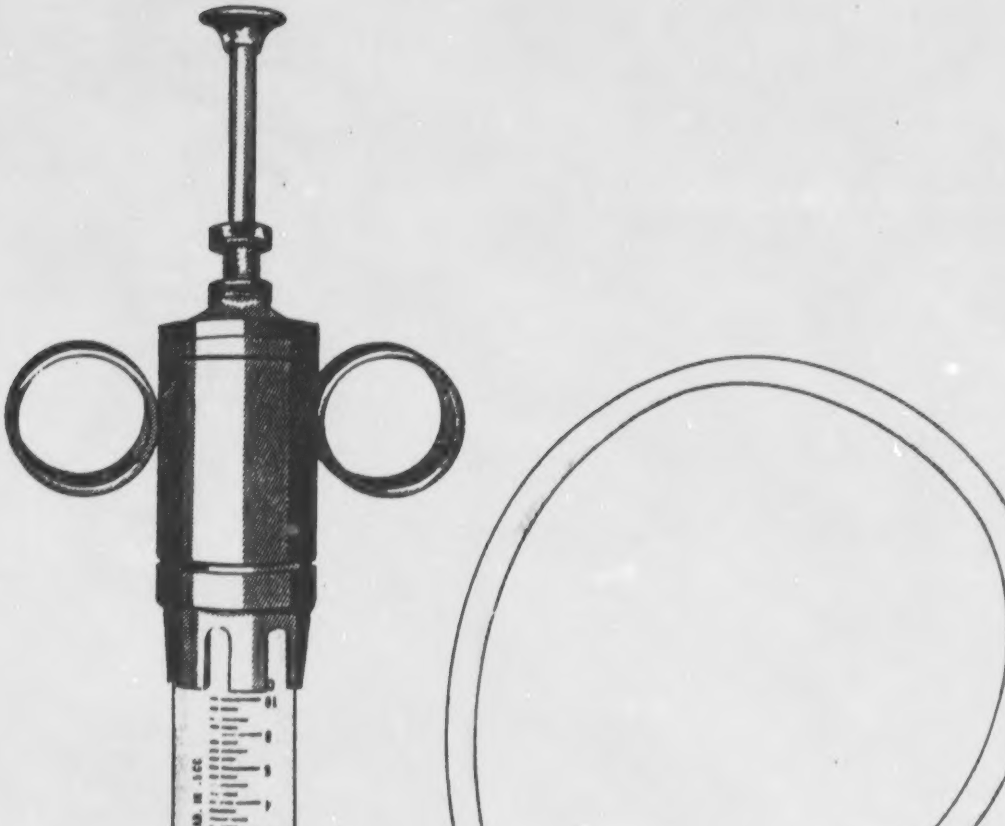


Figure 29. Automatic syringe.

ume. An automatic device is recommended because it is quicker, and consistent volumes are dispensed.

9-35. How you distribute medium aliquots will depend upon the use to which you put the aliquots. Petri dishes give a large surface area on which to spread a clinical specimen so that you can get isolated colonies and pure cultures. Test tubes allow a small amount of medium to be used for such studies as motility, biochemical, and physiological testing. On occasion you may need a large volume of fluid medium for a culture which needs a long incubation period. A blood culture falls in that category. You can use screw-capped bottles of assorted sizes to hold liquid or solid medium.

9-36. After using a dehydrated medium, make sure that you have replaced the cap tightly on the stock bottle. This tight closure prevents absorption of moisture from the air and keeps the medium in powdered form. Store the bottles in a cool, dry place away from windows and heating equipment.

9-37. *Rules for prepared media.* Once you have prepared and poured the medium and it is ready for storing, you must observe the following rules:

a. Most commonly used media should be refrigerated.

b. Thioglycollate broth and semisolid media should be stored at room temperature. If the thioglycollate broth shows a pink or red layer at the surface, heat the tubes of media in a hot water bath before inoculation to drive off the excess oxygen before use. Heat the tubes only once; additional heatings make the broth unsuitable for bacterial growth.

c. All plates should be incubated at room temperature in an inverted position for at least 18 hours or overnight to check for sterility.

d. When removed from the refrigerator, plates should be allowed to warm to room temperature before use. Inoculation of a cold medium lengthens the lag phase of the growth curve and may even kill some organisms.

e. Media containing a dye should be protected from light during storage because some dyes break down under strong light.

f. Condensation of moisture on tubes and petri dishes due to refrigeration is likely to increase the chance of contamination of the medium in those particular tubes and dishes. Surface areas of media should be moist (not wet or dry) for best results. A wet surface makes it difficult to obtain isolated colonies and heightens the chances of contaminating an area of the laboratory while you are streaking the plate with a wire loop.

g. If media are to be stored for more than 5 days, tubes or plates must be protected from dehydration by enclosing them in suitable containers such as a plastic bag. A dried medium, as manifested by the medium retracting from the edge of the tube or plate, or by a dry, dull surface, should not be used because inaccurate results and abnormal growth will result.

9-38. **Media Additives.** There are many different reagents which you can add to media for special purposes. Some reagents neutralize antibiotics which might be present in the specimen from a patient being treated for an infection; others promote growth of specific organisms having unusual nutritional needs; still others give instant identification of bacteria by color. We will take a look at some examples that you may run across in your work in bacteriology.

9-39. *Para-aminobenzoic acid.* This substance neutralizes the bacteriostatic effect of sulfonamides in culture media. Any medium that is inoculated with a sulfonamide-containing specimen may require the addition of 0.0002 percent para-aminobenzoic acid (PABA). If para-aminobenzoic acid is not available, you may use a similar concentration of procaine hydrochloride. This requirement for PABA particularly pertains to urine, blood, and fecal cultures from patients who may have received sulfonamide therapy.

9-40. *Sodium thioglycollate.* This is incorporated in media not only to permit the growth of anaerobic bacteria (because of its reducing action) but to neutralize the bacteriostatic effect of mercurial and silver disinfectants. Mercury compounds are often used as preservatives in plasma, serum, biologicals, and pharmaceuticals intended for parenteral injection. You should use thioglycollate media to test the sterility of such products.

9-41. *Antibiotic inhibitors.* These are substances which neutralize the activity of antibiotics. They are added to culture media when clinical material is suspected of containing the corresponding antibiotics. For instance, you use the enzyme penicillinase to inactivate penicillin, and also to inactivate streptomycin to some degree. In culturing blood or other body fluids containing penicillin, you should add 1 ml. of penicillinase to each 100 ml. or less of sterile medium. This amount of the enzyme will inactivate 50,000 units of penicillin, the highest level of antibiotic that could be expected in 10 ml. of blood or other body fluids. One ml. also inactivates up to 1,000 units of streptomycin. Penicillinase does not interfere with the sulfa-inactivating action of para-aminobenzoic acid which may also be added to the medium. Keep penicillinase refrigerated during storage, because this reagent, like many enzymes, loses activity at room temperature.

67

9-42. *Dyes.* Dyes which are incorporated in media have selective bacteriostatic effects, but they are generally more active against gram-positive bacteria. Crystal violet, brilliant green, and basic fuchsin are used in well-known selective media. Thionin and basic fuchsin are used in differentiating species of *Brucella.* If you are trying to isolate streptococci in the presence of staphylococci, the latter can be effectively inhibited if you add a 1:500,000 dilution of crystal violet to the broth or agar media. Add one milliliter of autoclaved aqueous 1:25,000 solution of the dye to each 20 ml. of medium.

9-43. *Sodium desoxycholate and other bile salts.* In proper combination with other substances in the media, they inhibit the growth of Gram-positive bacteria. The addition of this inhibitor is useful when we try to isolate Gram-negative bacteria from clinical material in which both Gram-positive and Gram-negative bacteria are present. On desoxycholate agar the motility of all flagellated bacteria is temporarily suppressed, and the swarming of certain species of *Proteus* is considerably inhibited. If citrates are also present, as in desoxycholate-citrate agar or SS agar, the bacteriostatic effect extends to some of the Gram-negative bacteria, notably the nonpathogenic enteric bacilli. Bile salts are not used successfully in media to isolate the gonococcus, meningococcus, or species of *Hemophilus.*

9-44. *Potassium tellurite.* When added to culture media in a final concentration of 0.01 percent, this retards the growth of most Gram-negative bacteria and permits the growth of *Streptococcus* species and other Gram-positive organisms. A 0.03-percent concentration of tellurite will inhibit most *Streptococcus* species, but *Staphylococcus* species and the Corynebacteriae are still able to grow. Potassium tellurite is most useful in agar for the isolation of *Corynebacterium diphtheriae.* You can add it to broth to isolate Gram-positive bacteria, notably *Staphylococcus* species from mixed culture material overgrown by species of *Proteus* or other rapidly growing Gram-negative bacilli.

9-45. *Chloral hydrate.* If added to nutrient agar in a final concentration of 0.1 percent, it has little or no inhibitory effect on either Gram-positive or Gram-negative bacteria. It does, however, prevent the swarming of species of *Proteus,* rendering them temporarily nonmotile. Unlike bile salts and tellurite, chloral hydrate does not "lake" blood (lyse red blood cells) and so you may use it in blood agar plates without interfering with the development of characteristic zones of hemolysis by streptococci and other organisms.

9-46. *Media containing antibiotics.* In appropriate concentrations, these media are inhibitory to some organisms and noninhibitory to others. Antibiotic media may be used in the selective isolation of certain pathogens from specimens containing many contaminating microorganisms. For example, you may incorporate penicillin in Bordet-Gengou agar in a concentration of 1 unit per ml. of medium. The antibiotic will restrict growth of some Gram-positive organisms of the throat and allow isolation of *Bordetella pertussis,* the cause of whooping cough. Penicillin and streptomycin in combination, or chloramphenicol alone, may be incorporated in media for the isolation of certain fungi from bacteria-contaminated specimens.

9-47. *Sodium azide.* This additive, which inhibits the growth of Gram-negative organisms, is also used in culture media to help isolate hemolytic *Streptococcus* species from the respiratory passages. Special-purpose broth containing glucose (0.02 g. to 100 ml. of broth) is tubed in 2-ml. amounts. On the day the medium is to be used, 0.15 ml. of a sterile aqueous solution of sodium azide (1 to 1,000) and 0.1 ml. of a sterile aqueous solution of crystal violet (1 to 25,000) are added to each tube. Place material obtained by swabbing the throat or nasal passages in the azide broth within 1 hour after collection. After overnight incubation of the broth, make subcultures on blood agar plates. The sodium azide effectively inhibits growth of staphylococci and permits the hemolytic streptococci to form colonies.

9-48. **Biochemical Media.** There are a wide variety of media to test for biochemical reactions. These reactions are valuable tools for getting more specific identifying information on an organism isolated in pure culture. We will point out the reasons for using biochemical media and cite examples of the following:

a. Differential carbohydrate media.

b. Carbohydrate fermentation media.

9-49. Carbohydrates, including many of the 6-carbon and 12-carbon sugars such as glucose and sucrose, respectively, are excellent sources of energy for microorganisms. However, there are great differences in the ability of bacteria, though closely related taxonomically, to use one or more of these numerous compounds for energy and growth. This feature is particularly helpful to you in distinguishing among the morphologically similar facultative anaerobes which inhabit the human intestinal tract—for example, the coliform bacilli, the typhoid organism, and the salmonellas of food infection.

9-50. Not only does the ability of an unknown organism to ferment glucose but not lactose tell

74

you a great deal, but the fact that the breakdown of sugars is usually accompanied by the formation of identifiable byproducts is also important. That is, you can test for the production of organic acids and certain gases which form in the culture during breakdown of a carbohydrate medium. Two differential media commonly used to distinguish between closely related microbes, Kligler's iron agar (KIA) and triple sugar iron agar (TSI), are useful because they take advantage of fundamental differences in the physiology of bacteria that seem identical from outward appearances.

9-51. KIA contains lactose, dextrose, nutrient agar, and phenol red indicator. TSI contains sucrose in addition to lactose and dextrose. Color changes in these media, caused by selective fermentation of the carbohydrates, form the basis for genus and species identification. KIA and TSI also contain an iron compound which indicates whether or not an organism can produce hydrogen sulfide (H_2S). As fermentation proceeds, the medium is blackened through the reaction of H_2S and iron to form iron sulfide.

9-52. The study of reactions in KIA and TSI differential media usually leads only to tentative or presumptive identification of an organism. Identity must be confirmed by other means such as the fermentation of additional sugars and sugar alcohols, or by serological procedures. This situation comes about because bacteria belonging to related but different genera sometimes give identical results in one or more of the reactions seen in KIA and TSI media. For example, some species of *Shigella, Aerobacter, Escherichia,* and *Proteus* yield both a yellow slant and butt in a TSI culture tube. The presence of gas bubbles and H_2S production can often be used as distinguishing characteristics, but in general, reaction on differential carbohydrate media serves as a preamble to further steps in identification.

9-53. To study carbohydrate fermentation by bacteria, you must choose a proper basal medium. One broth base which you can use is made up of pancreatic digest of casein and sodium chloride dissolved in water with no carbohydrate present. Add the appropriate test carbohydrate (e.g., sucrose, mannitol, inulin) and an indicator such as bromcresol purple or phenol red, and the pH is adjusted. The medium must have a specific pH to detect carbohydrate fermentation. In the presence of an indicator, a change of pH due to accumulation of bacterial products gives a visual indication of fermentation. Specific reactions on differential and carbohydrate media will be shown later in our study of individual genera of the pathogenic microorganisms.

9-54. **Biochemical Tests.** Over the years, many techniques have been developed to give information beyond that gained by observing growth on differential carbohydrate media such as Kligler's and triple sugar iron agar. For the most part, however, these techniques still depend on the detection of breakdown products of protein and carbohydrate metabolism, or oxidation-reduction reactions and fermentation patterns typical of some bacteria but not others. The procedures discussed in the following paragraphs are representative of the special tests commonly used.

a. Indole production.
b. Nitrate reduction.
c. Methyl red and Voges Proskauer test (MR-VP).
d. Proteolytic activity.
e. Milk fermentation.

9-55. *Indole production.* This procedure is based on the ability of certain organisms to split the amino acid tryptophan to alanine and indole. You can detect the liberated indole by adding Kovac's reagent (para-dimethyl-amino-benzaldehyde). A red color indicates positive indole production. There are several media available for this purpose, but in all cases, be certain that tryptophan (in the form of peptone) is present in the medium.

9-56. *Nitrate reduction.* Many bacteria are able to reduce *nitrate* to *nitrite* (NO_3NO_2), a property which helps us identify them. Use a broth or solid agar medium which contains a source of nitrate, such as sodium nitrate ($NaNO_3$). Incubation will produce frothing in the broth medium and cracks in the agar as a result of nitrate reduction and subsequent nitrogen gas production. Add sulfanilic acid and dimethyl-alpha-naphthylamine to develop the pink or red color which indicates the presence of nitrites.

9-57. *Methyl red and Voges Proskauer (MR-VP) tests.* These reactions are used frequently to differentiate certain bacteria of the enteric groups. The methyl red test is used to identify organisms that ferment dextrose and produce acids as final end products. The test is based upon a change in the pH of Clark and Lub's broth. An indicator dye, methyl red, turns the broth a yellow color when the pH is greater than 4.5. This is a negative MR test. A pH of 4.5 or less will turn the solution a distinct orange-red or red. This is a positive test.

9-58. The Voges Proskauer test is based on the ability of some organisms to produce a neutral end product, acetyl-methyl-carbinol, from an acid resulting from dextrose fermentation. Dextrose is fermented to pyruvic acid to acetaldehyde to acetyl-methyl-carbinol. A few drops of 5 percent alph-naphthol in absolute alcohol and 40 percent potassium hydroxide are added to a broth culture. A positive test develops a copper or red color. A yellow color is regarded as a negative test.

9-59. *Proteolytic activity.* This activity of bacteria is another aid to their individual identification. Some bacteria produce enzymes which break down protein and protein derivatives. For example, they can liquify gelatin and cause coagulated egg albumin to become fluid again. You can use gelatin liquifaction to detect this proteolytic activity in the laboratory, but you can also use coagulated serum as a test medium. Gelatin liquification is frequently used to distinguish the genus *Aerobacter* from other members of the family *Enterobacteriaceae*, primarily the genus *Klebsiella*. Coagulase-positive *Staphylococcus* species usually liquify gelatin.

9-60. *Milk fermentation.* The changes in a milk medium caused by bacterial action take several forms. Lactobacilli, or *Streptococcus lactis*, sour milk by producing lactic acid. These organisms are used commercially to prepare buttermilk, sour cream, yogurt, and other dairy products. Gas and unpleasant flavors are produced by *Escherichia coli* or other coliforms which ferment milk with less acidity. Our concern is with the reaction of the coliform bacteria.

9-61. Fresh milk has a pH of approximately 6.8. As an empirical or natural medium, milk is ideal for culturing many microorganisms. If you add indicators such as litmus or bromcresol purple to skim milk, you can detect the changes taking place during fermentation. Litmus milk has a differential value based on lactose fermentation, reduction of litmus, coagulation of milk proteins, and digestion of the casein. You can determine different degrees of acidity (lactose fermentation) or alkalinity by the color of the indicator. Reduction of litmus is shown by its decolorization.

9-62. Coagulation is seen as clotting of the milk (curd formation) due to the accumulation of acid, which produces a firm clot that does not shrink. If lactose is not fermented at all, or ferments slowly, clotting may also result from the action of a rennetlike enzyme which forms a soft clot unaccompanied by marked acidity. Digestion of the casein, indicated by a partial or complete clearing of the milk, may or may not be preceded by coagulation.

9-63. Stormy fermentation is a term used to describe a reaction in milk produced by *Clostridium perfringens (Welchii)*, one of the organisms that cause gangrene. The reaction is the result of rapid coagulation of milk proteins followed by active gas production which results in tearing of the clot. This reaction is not as specific for *Cl. perfringens* as was formerly supposed, and some strains of the organism produce this effect slowly and only under special conditions.

9-64. There are many types of media, each with a variety of uses. The preceding paragraphs have given you a basic explanation of how certain reactions take place and their role in identification of bacteria. As we proceed with the discussion of various bacteria and their growth and identifying characteristics, we will discuss other special media or additional tests as they are needed. For you who are interested, there are many text books available to you in medical libraries and clinical laboratories which detail the biochemistry and metabolism of bacteria in various environments.

Inoculation of Media for Isolation of Bacteria

LITTLE PROGRESS was made in associating bacteria with specific diseases until Robert Koch devised solid culture media well after the midpoint of the 19th century. This was discussed briefly in Chapters 1 and 2. Thus, nearly 200 years had elapsed before Koch's pure culture techniques permitted a systematic study of the single-celled organisms which Van Leeuwenhoek observed in his microscope in 1676.

2. As soon as means were available to isolate individual bacteria and cultivate them in the laboratory, advances in the knowledge of infectious diseases came rapidly. In 1877 Koch proved the causal relationship between *Bacillus anthracis* and anthrax in domestic animals. Other pathogens were isolated in quick succession during the 1880s by Koch and other investigators who had adopted his methods: the tubercle bacillus, the cholera vibrio, the pneumococcus, and the etiologic agents of meningitis, diphtheria, and tetanus.

3. The major problem in diagnostic bacteriology of that era is the same problem we face today in the clinical laboratory—how to separate pathogenic microbes from harmless organisms with which they are closely associated in nature. From the moment of birth the human body is heavily populated with bacteria representing many different genera. Some of these microbial populations are *transient*, but others are *resident* in the sense that they can almost always be recovered in large numbers from the skin, the body orifices, and the intestinal tract—the so-called normal flora.

4. As Koch demonstrated many years ago, the essential first step in identifying an organism is to segregate it from the varied mixed populations found within our bodies, in soil, in water, in air and on objects in our external environment. Once a single-celled microorganism is immobilized on a solid culture medium suitable for growth and reproduction, its progeny provide us within the space of a few hours the material needed for cultural studies—an isolated colony visible to the naked eye. Beyond this point, different procedures developed over the years for subculturing the colony enable us to select the conditions of growth best suited to bring out the morphological and physiological characteristics which experience has shown will establish the identity of unknown organisms.

5. Long before Koch introduced the concept of pure cultures, other researchers, notably Ehrenberg in the 1830s, began the monumental task of classifying bacteria on the basis of their microscopic appearance. His work was carried on under the severe handicap of having to observe single-celled organisms in their natural state, that is to say, without the aid of dyes to improve the resolution of cell shape, size, and internal structures. Koch made a second great contribution to the science of bacteriology by developing staining procedures to accentuate the morphological features of bacteria seen under the light microscope. Today, as we know, staining techniques not only furnish an invaluable means of tentative identification but also serve as a guide to the culture methods which we must choose to confirm the identity of an unknown microorganism.

10. Isolation and Cultivation of Bacteria

10-1. In almost every case the pus, urine, stool or other clinical specimen submitted to the laboratory contains more than one kind of organism. The organisms may all be pathogens, or one or more pathogens may be found among several saprophytes. There are several ways of obtaining pure cultures from mixed populations, although more than one method may have to be used in processing a single specimen. For example, filtration of a large volume of liquid may precede the plating of material extracted from the liquid. The choice of methods, or combination thereof, depends upon the nature of the specimen. These are the commonly used procedures:

- Plating (streak or pour plates).
- Subculturing.

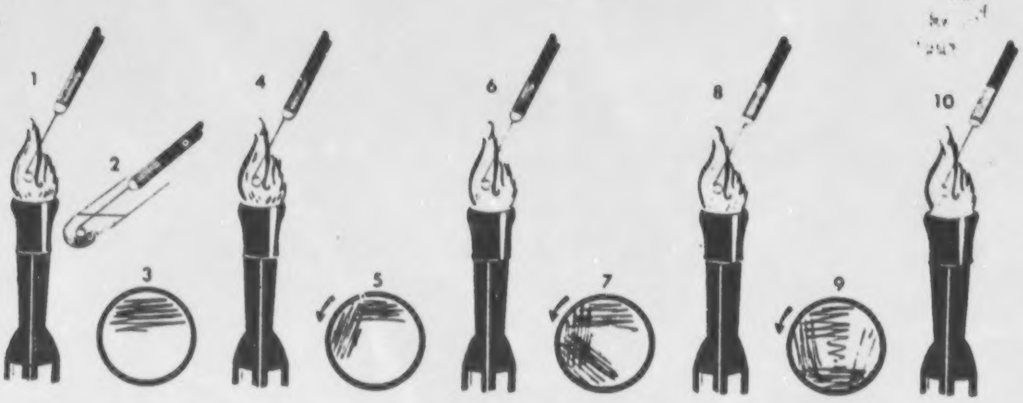


Figure 30. Procedure for streak plate.

- Dilution.
- Filtration.

10-2. **Plating.** Streaking an agar surface to isolate bacteria is routine at one point or another with almost any specimen received in a bacteriology laboratory. But to be successful in separating organisms in a mixed population so that individual pure colonies grow on the surface, you must follow certain basic techniques.

10-3. *Streak plate.* The streak plate technique uses glass or plastic dishes (petri plates) containing a solidified nutrient agar. Streaking is accomplished in the sequence of steps shown in figure 30. Collect a loopful of inoculum on a flame-sterilized wire loop needle and streak it over approximately one-quarter of the agar surface. After flaming the loop again, rotate the plate slightly and streak another quadrant of the agar surface. Notice in the second step (fig. 30) that the loop passes through an area of the plate that has just been inoculated, picking up organisms deposited by the needle in its earlier sweeps across the surface. Continue this process of diluting and spreading the inoculum over the medium until the entire agar surface is covered. As you continue the streaking, fewer and fewer cells are dispersed by the loop, and finally single cells are spread across the agar. Each cell will grow into a visible colony under suitable environmental conditions. If you are culturing clinical material on a cotton swab, you can roll the swab over a small area of the agar surface at the edge of the plate. Then, spread the inoculum with a wire loop as just described.

10-4. Although many agar base media are available for your use, a blood-enriched peptone agar is usually the medium of choice for the primary isolation of fastidious pathogenic bacteria. As a routine matter, the streaked plates are incubated at 37° C. for 18 to 24 hours in the inverted position, followed by examination of isolated colonies, grossly and microscopically, for characteristics of various genera and species. You can "pick" pure cultures by touching the center of a colony with a wire needle and subculturing the material to suitable broth or agar media for additional tests.

10-5. Remember, however, that several organisms may clump together during inoculation of a specimen to an agar plate. The resulting growth will be a mixed colony which is often difficult to recognize as such. Bacteria of different species will obviously produce an impure colony, yielding atypical results in biochemical tests employed for identification purposes. This situation points up the value of studying a stained slide of the growth at the time the colony is picked for further tests. If the slide reveals that the colony consists of more than one type of bacteria, you must either select a different colony from the plate or else restreak the colony originally chosen. Restreaking from one plate to another is one form of *subculturing* to obtain isolated growth of mixed flora.

10-6. *Pour plate.* The *pour plate* technique for securing isolated colonies also generally involves blood agar. Culture tubes of sterile infusion agar or other suitable basal medium are first melted in boiling water. After cooling the medium to about 48° C. in a water bath, add sterile defibrinated blood aseptically. Then, inoculate each tubed blood-medium mixture with a sample of the specimen or broth culture, mix gently and dispense it to a sterile petri dish. Since your specimen inoculum may hold too many microbes to yield widely spaced colonies on the pour plate, it is a good idea to prepare a second plate containing a dilution of the inoculum. You make this dilution by mixing a loopful of material from the specimen in 5 or 6 ml. of sterile broth or saline.

Then add one loopful of this diluted specimen to a tube of blood medium, and pour the medium into a sterile petri dish as before.

10-7. When the medium solidifies, individual cells of the inoculum are immobilized in various areas of the agar. During incubation each cell will multiply to form a visible colony. To make subcultures or prepare stained slides from pour plate cultures, pick growth from individual colonies within the agar using a sterile needle. The pour plate offers an advantage if you want to perform bacterial counts on various types of specimens; however, the pour plate procedure is more time consuming than is the case of streaking.

10-8. Because of the time factor, blood agar pour plates, as opposed to streak plates, are mostly used to determine the type of hemolysis produced by strains of streptococci. The "O" hemolysins of beta hemolytic streptococci are active only under anaerobic conditions such as those provided by the depths of the agar pour plate.

10-9. *Subculturing.* After you have obtained isolated colonies by the streak plate or pour plate technique, you usually need to transfer (subculture) them to tubed media for further study. There are several different configurations in tubed media, each developed for a specific purpose, but we will limit our discussion to the following types:

- Liquid broth cultures.
- Slant cultures.
- Stab cultures.

10-10. *Liquid broth cultures.* These are used to maintain the viability of an organism by successive transfers to fresh medium; to grow a large volume of liquid culture for inoculation to differential or fermentation media; or to study motility. To inoculate a broth culture, use a wire loop to pick a colony from a plate. Emulsify this material on the moist wall of the tube just above the liquid level, and wash down the solid matter by tilting the tube. If the inoculum is liquid instead of growth from a colony, simply place a loopful in the broth and disperse it by gentle agitation. A word of caution, though: if you swirl the loop too vigorously, droplets will escape from the tube and contaminate the surrounding air. The resulting aerosol can infect you and your fellow workers. Don't forget, too, when transferring broth cultures with a pipette, to use a cotton plug in the mouthpiece to prevent accidental contamination of your lips and mouth.

10-11. *Slant cultures.* Slant cultures are used for biochemical tests as well as to maintain stock cultures in the laboratory. We learned earlier in Chapter 3 that "slants" are tubed agar media that have been allowed to harden in a near-horizontal position to give a large surface area for bacterial growth. You prepare them by streaking an inoculum over the slant surface from bottom to top. If the slant contains water or condensation at its base, drain the moisture out by inverting the tube before inoculation. Do not spread the water over the surface of the slant because the resulting growth will not give a characteristic appearance.

10-12. *Stab cultures.* These are also used in biochemical tests, especially for anaerobic organisms. You can make stabs with a straight needle into tubes of solid or semisolid medium (not slanted) which hardened in a vertical position to give great depth and a small surface area. You should center the stab line, and extend it approximately two-thirds of the depth of the medium. Insert the inoculating wire straight and evenly to avoid tearing the medium and creating bubbles. Later, these bubbles could be mistaken for gas production. In some instances, the features of stab and slant cultures are combined. For instance, the differential medium, triple sugar iron agar, described in the preceding chapter is both a slant and stab culture. It is used to detect sugar fermentation as well as H_2S production in the same tube.

10-13. **Diluting.** In the description of a pour plate technique we touched on the advisability of diluting the inoculum from a clinical specimen to insure that well isolated colonies would grow out on the plate. You can also use dilution cultures to estimate the number of bacteria in a specimen. A urine sample, for instance, may contain so many bacteria that it would be impossible to obtain separate and pure colonies by plating the undiluted urine. Diluting this specimen, say 1:100 and 1:1000 in saline, not only assures isolation of colonies, but allows you to make a colony count. Multiplying the number of colonies on the plate by the dilution factor tells you the number of bacteria per ml. of urine. In this calculation, each colony is assumed to be the progeny of a single bacterium present in the original specimen.

10-14. **Filtering.** Filter cultures are a means of cultivating bacteria on a solid surface without using specific solidifying agents. A technique developed by the Millipore Filter Corporation[3] uses a very thin, highly porous membrane filter made of cellulose acetate, colloidon, or similar material. First remove bacteria from liquid specimens passed through the filter. Then place the membrane holding the trapped bacteria on a conven-

---

[3] The apparatus and technique for its use are described more fully in ADM-40, *Techniques for Microbiological Analysis*, 1963 edition. Millipore Filter. Corp. Bedford, Mass.

MODIFICATIONS

Figure 31 of this publication has(have) been deleted in adapting this material for inclusion in the "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education." Deleted materials are copyrighted and could not be duplicated for use in vocational and technical education.

tional agar medium plate as you see in figure 31, and the bacteria will grow to colony size right on the membrane which absorbs nutrients from the medium. Figure 32 shows typical growth of colonies on the filter after incubation. Filters of this type can be adapted to many uses, but they are particularly advantageous under conditions in which you must collect and identify a few bacteria dispersed in a large volume of air or water.

11. Initial Isolation Procedures

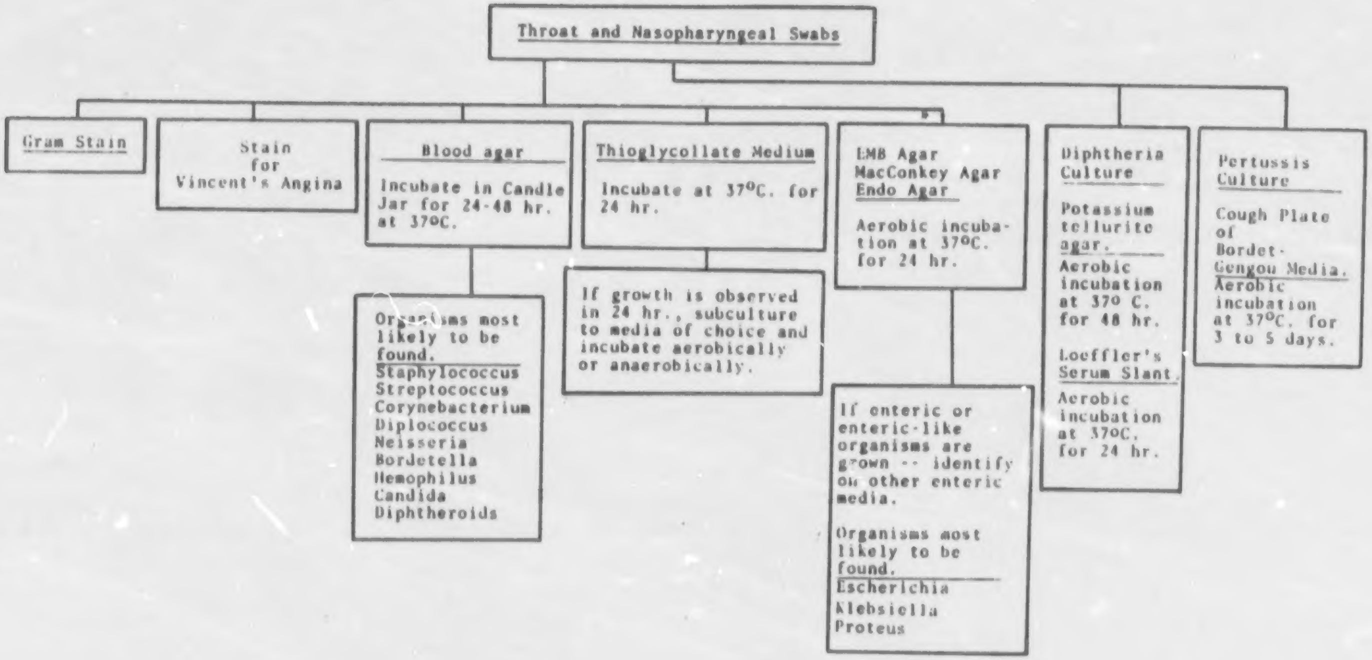
11-1. Specimens from many sources containing a wide spectrum of bacteria constitute the workload of a bacteriology section. In setting up cultural conditions favorable to isolation of the diverse species found in clinical material, you should keep in mind the growth parameters that we discussed in the previous chapter. Incubation temperature, pH, moisture, the proper gaseous atmosphere, and suitable nutrients all come into play in establishing an ideal environment for propagation of pathogens in the laboratory. In this chapter we will present a flow chart for each type of specimen, outlining the primary isolation media and noting the organisms usually supported by each medium. Specific reactions for identifying the organisms will be discussed in greater depth in a later chapter of this volume and in Volume 2.

11-2. **Throat and Nasopharyngeal Swabs.** The selection of media for culturing throat and nasopharyngeal specimens must be broad because so many different genera may be involved. In figure 33 we have diagrammed a flow chart to follow in working your cultures. The pathogenic organisms most often found in the throat and nasopharyngeal area include species of beta hemolytic streptococci, *Staphylococcus aureus, Corynebacterium diphtheriae, Borrelia vincentii, Hemophilus*, and *Bordetella* species. The genus *Bordetella* now includes *B. pertussis*, which formerly was classified as *Hemophilus pertussis*.

11-3. For routine cultures, inoculate a blood agar plate by either the streak or pour plate method, generally the former. Blood agar differentiates between the several types of hemolysis (lysis of red blood cells around the colony) and furnishes the nutrients required by many fastidious pathogenic bacteria. Incubate the plate at 37° C. in a candle jar for 24 to 48 hours to provide a slightly higher than ambient carbon dioxide (CO_2) environment. The increased CO_2 is not harmful to the other pathogens and is required for isolating *Neisseria meningitidis*, the agent of epidemic meningitis. Chocolate agar (heated blood) is preferred by some strains of

MODIFICATIONS

Figure 32 of this publication has(have) been deleted in adapting this material for inclusion in the "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education." Deleted materials are copyrighted and could not be duplicated for use in vocational and technical education.


Throat and Nasopharyngeal Swabs
Gram Stain
Stain for Vincent's Angina
Blood agar
Incubate in Candle Jar for 24-48 hr. at 37°C.
Organisms most likely to be found.
Staphylococcus
Streptococcus
Corynebacterium
Diplococcus
Neisseria
Bordetella
Hemophilus
Candida
Diphtheroids
Thioglycollate Medium
Incubate at 37°C. for 24 hr.
If growth is observed in 24 hr., subculture to media of choice and incubate aerobically or anaerobically.
*
EMB Agar
MacConkey Agar
Endo Agar
Aerobic incubation at 37°C. for 24 hr.
If enteric or enteric-like organisms are grown -- identify on other enteric media.
Organisms most likely to be found.
Escherichia
Klebsiella
Proteus
Diphtheria Culture
Potassium tellurite agar.
Aerobic incubation at 37° C. for 48 hr.
Loeffler's Serum Slant.
Aerobic incubation at 37°C. for 24 hr.
Pertussis Culture
Cough Plate of Bordet-Gengou Media.
Aerobic incubation at 37°C. for 3 to 5 days.

*Optional -- may save time in identifying coliforms.
-- especially useful when doing cultures from children.

Figure 33. Flowchart for processing throat and nasopharyngeal swabs.

N. meningitidis, so in addition, streak a plate of chocolate agar, in cases of suspected meningitis.

11-4. It is a good idea to make two or three slanted cuts in the agar with a loop in the heavily inoculated area of the streak plate. This will allow subsurface growth of streptococci which produce the "O" hemolysin under anaerobic conditions. Another technique to detect the hemolysin is to place a sterile coverslip on the surface of the streaked plate, over the slanted cut. The medium beneath the coverslip is devoid of atmospheric oxygen, permitting the "O" hemolysin to form. You have already learned that pour plates, as well as streaked plates, are often used in studies on streptococcal hemolysins.

11-5. You should inoculate a part of the specimen into thioglycollate broth, an excellent general-purpose medium which supports the growth of both aerobes and anaerobes. Before you inoculate the tube of thioglycollate, check to see whether or not the broth has changed color. The normal bluish color is due to an indicator, methylene blue, which turns pink when atmospheric oxygen diffuses into the medium. If the pink color is seen *below* the upper one-third of the tube, you must heat the medium in boiling water to drive off dissolved oxygen and then quickly cool it. Do not reheat the tube more than once, because repeated heatings alter the chemical composition of the thioglycollate broth.

11-6. The type of growth in the thioglycollate medium (sediment, pellicle, floccules) gives a hint of the next step to take in identification. You should then subculture organisms seen in the broth and incubate them further under aerobic or anaerobic conditions, depending on the level at which the growth appeared within the thioglycollate medium. A pellicle on the surface shows the presence of an aerobe. Conversely, growth deep in the tube must have been formed by an anaerobe, so you should incubate subcultures anaerobically in this instance. The Gram reaction and morphology on a stained slide of the bacteria in thioglycollate medium will suggest the type of medium and atmospheric environment to be used in subculturing.

11-7. Inoculate a part of the throat or nasopharyngeal specimen to a slant of Loeffler's serum medium and potassium tellurite medium if *Corynebacterium diphtheriae* is suspected. The organism grows out on Loeffler's in 18 to 24 hours at 37° C. You should allow the potassium tellurite agar to incubate at 37° C. for 48 hours to allow the formation of the gray to black colony pigment characteristic of *C. diphtheriae*. If the physician wants to rule out whooping cough, he may ask for a "cough" plate of Bordet-Gengou medium. By this technique, the patient coughs directly onto the surface of the plated medium, and *Bordetella pertussis* expelled in saliva droplets appears as isolated colonies on the surface of the selective medium.

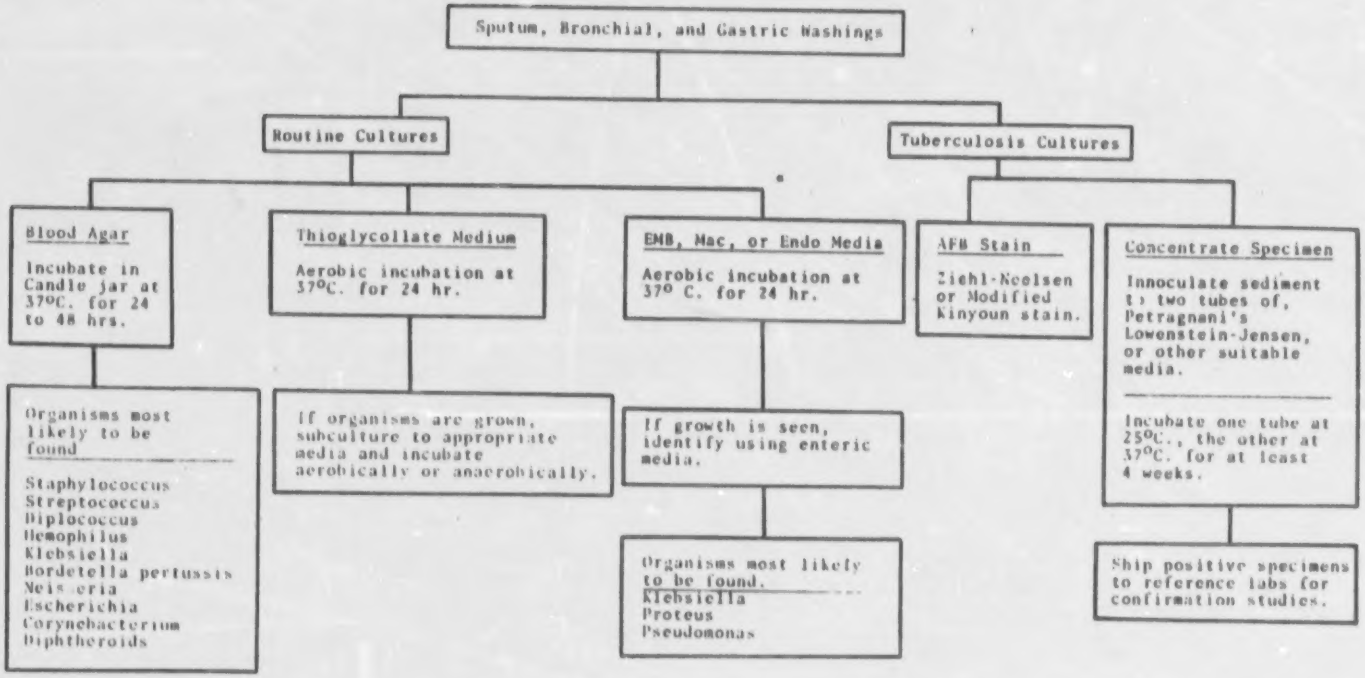
11-8. If you are looking specifically for staphylococci, it helps to streak a medium containing mannitol and a high concentration of sodium chloride in an effort to isolate pathogenic *Staphylococcus aureus* in minimum time. A medium such as mannitol salt agar is selective in this respect. Colonies which produce a yellow discoloration of the medium are mannitol fermenters. The fact that growth has occurred in a high salt concentration is presumptive evidence that the organism belongs to the genus *Staphylococcus*. You must necessarily follow with confirmatory tests as to genus and species.

11-9. One of the enteric media such as eosin-methylene blue, MacConkey, or Endo agar will aid in isolating Gram-negative bacilli that may be present in the throat. Recovery of *Proteus* spp. and *Escherichia coli* from throats of young children is common.

11-10. As part of the initial processing, make a direct slide smear from the specimen, particularly if Vincent's Angina (Trench Mouth) is suspected. It is best to use a separate swab, but if only one swab was received, prepare the smear *after* the culture media are inoculated. As a rule, glass microscope slides are not sterile. If slides are smeared with the swab before the plates are inoculated, there is a good chance that the media will be contaminated by organisms which were not present in the specimen.

11-11. **Sputum, Bronchial, and Gastric Washings for Acid-Fast Bacteria (AFB).** You can find a wide variety of organisms, some of them highly infectious, in cultures of these specimens. In figure 34, we have shown a laboratory processing scheme for successfully isolating representative microorganisms. The specimen is usually derived from a true or suspicioned case of pneumonia or tuberculosis. Generally, the physician will specify that he wants "acid-fast" or tuberculosis studies. Otherwise, you set up routine procedures that will grow out bacteria other than *Mycobacterium tuberculosis*, which requires special processing steps. Organisms most likely to be isolated from sputum and other specimens in this category are *Staphylococcus aureus, Streptococcus spp., Diplococcus pneumoniae, Klebsiella pneumoniae, Hemophilus spp., Bordetella pertussis*, the yeast-like *Candida spp.*, and *Mycobacterium tuberculosis.*

11-12. You can do practical and efficient isolation of these organisms, except for the tubercle bacillus, by inoculating blood agar, enteric media and thioglycollate broth in accord with the cul-



Sputum, Bronchial, and Gastric Washings

Routine Cultures

Tuberculosis Cultures

Blood Agar
Incubate in Candle jar at 37°C. for 24 to 48 hrs.

Organisms most likely to be found
Staphylococcus
Streptococcus
Diplococcus
Hemophilus
Klebsiella
Bordetella pertussis
Neisseria
Escherichia
Corynebacterium
Diphtheroids

Thioglycollate Medium
Aerobic incubation at 37°C. for 24 hr.

If organisms are grown, subculture to appropriate media and incubate aerobically or anaerobically.

EMB, Mac, or Endo Media
Aerobic incubation at 37° C. for 24 hr.

If growth is seen, identify using enteric media.

Organisms most likely to be found.
Klebsiella
Proteus
Pseudomonas

AFB Stain
Ziehl-Neelsen or Modified Kinyoun stain.

Concentrate Specimen
Innoculate sediment to two tubes of, Petragnani's Lowenstein-Jensen, or other suitable media.

Incubate one tube at 25°C., the other at 37°C. for at least 4 weeks.

Ship positive specimens to reference labs for confirmation studies.

*Optional -- may save time identifying coliforms.

Figure 34. Flowchart for processing sputum, bronchial and gastric washings.

tural conditions we established for throat and nasopharyngeal swabs. You should also prepare a smear to check visually for bacteria in the specimen. Remember, a stained smear gives only a rough indication of the number and kind of organisms present. If the stain is done properly, however, the size, shape, and Gram reaction of the microbes will serve as a guide to subsequent culture procedures.

11-13. For acid-fast studies (AFB) you need special media, and the sputum specimen, itself, is handled differently. If a routine culture is requested in addition to a culture for tuberculosis (TB), you should set up the routine work before you start TB processing, or else you must use two separate specimens. This is necessary because the drastic digestion and concentration technique for isolation of the tubercle bacillus destroys other microorganisms in the sputum.

11-14. *Collection.* A sputum (not saliva) specimen must be collected either early in the morning or over a 12 to 24 hour period (never longer) to produce a representative sample. Gastric specimens should also be collected early in the morning from a fasting patient, and on three successive days. You must process stomach washings without delay because the highly acid gastric juices will inactivate the tubercle bacilli. Bronchial and gastric washings are collected only by a physician. Direct smears and concentration procedures should be instituted as soon as possible after collection of the specimens to insure recovery of viable acid-fast bacilli.

11-15. *Direct smear.* Tubercle bacilli from an active case are highly infectious. A hood is strongly recommended for processing possible tubercular specimens. It is also a wise precaution in handling sputum with a loop to dip the needle in phenol before flaming the tip to reduce contamination from "sputtering" as the specimen incinerates. In examining a sputum specimen select purulent, bloody, or caseous material to prepare direct smears. You may use either the Ziehl-Neelsen stain or the Modified Kinyoun, or both to stain the smear. Studies have shown that at least 100,000 bacilli per ml. of sputum must be present if we expect to find TB organisms microscopically. Regardless of whether acid-fast forms are seen on the slide, you should concentrate the sputum to increase your chances of isolating the tubercle bacillus in culture.

11-16. *Concentration.* There are several good concentration techniques which do not destroy the tubercle bacillus and which also reduce the risk of infection to the technician. Concentration techniques are based on the principle of breaking down tissue in which *Mycobacterium tuberculosis* is trapped, while at the same time destroying other, less resistant microbes which might later overgrow the tubercle bacillus during cultivation on isolation media. We are using sputum as a typical specimen in this discussion; however, the digestion and concentration techniques are equally applicable to bronchial and gastric washings.

11-17. Sodium hydroxide, trisodium phosphate, or commercially available enzyme preparations are used in the digestion process. Treatment of sputum samples with sodium hydroxide (NaOH) is one of the most widely used techniques of concentration. It is a simple procedure which involves: *first,* adding 4 percent NaOH in a volume equal to that of the specimen and shaking the mixture, preferably on a mechanical shaker, to homogenize the specimen. The *second* step consists of centrifuging the sediment and decanting the supernatant liquid into 5 percent phenol disinfectant. No later than 30 minutes after adding the NaOH, you should titrate the sediment to a neutral endpoint with hydrochloric acid (HCl). Portions of the sediment are then ready for preparation of additional slides, culturing, and animal inoculation.

11-18. The strong alkali, NaOH, offers three advantages: (1) it is quick, (2) it produces less sediment during digestion, and (3) it yields a neutralized sediment (after HCl treatment), which doesn't irritate animal tissues. This last factor is important in reducing the number of test animals which become sick or die as a traumatic result of the inoculation rather than from infection with the tubercle bacillus in the sediment.

11-19. Trisodium phosphate also has a digestive effect. It is especially useful for samples which cannot be processed immediately, for those which have large quantities of mucus, and for specimens that are to be mailed to a reference laboratory. Exposure to trisodium phosphate for 1 day, or longer, will digest the specimen and inhibit growth of microbes other than the TB organism, however, this reagent is less inhibitory than NaOH. Tubercle bacilli remain viable even after a week's exposure to trisodium phosphate.

11-20. You begin the process by mixing the specimen with an equal volume of 13 percent trisodium phosphate ($Na_3PO_4.12H_2O$) in a screw-cap bottle. Agitation on a mechanical shaker helps to speed up digestion. (Mailed specimens are usually digested by the time they reach their destination.) After you centrifuge and discard the supernatant, wash the sediment with sterile saline and centrifuge it again. Then the sediment is ready for slides and culture.

11-21. The n-acetyl-l-cysteine (NAC) technique is a relatively new enzymatic digestion procedure. Mucoproteins in sputum and mucus specimens are readily dissolved by a solution of the enzyme in weak NaOH. The main advantage of the NAC technique is greater survival of the tubercle bacillus during the digestion process because of the lower concentration of alkali present. If the sputum is very viscous, you can add a small amount of NAC powder directly to the specimen. Otherwise, add an equal volume of the digestant solution to the sputum in a tube and wait until the specimen clears (5 to 30 seconds). Let the mixture stand for an additional 15 to 30 minutes.

11-22. After digestion is complete, neutralize the NaOH with M/15 sterile phosphate buffer and centrifuge. Pour off the supernatant fluid into a disinfectant solution, being careful not to disturb the sediment. To this sediment add 1.0 ml. of bovine albumin (Fraction V) to serve as a buffer against pH change during growth of the culture. Mix well, inoculate culture media, and prepare slides for staining.

11-23. *Smear of concentrate.* You should examine air-dried, heat-fixed smears of the specimen and of the concentrated sediments for acid-fast bacilli. The previously mentioned Ziehl-Neelsen and Modified Kinyoun stains are widely used. Both use basic dyes in conjunction with other constituents that increase pentrability of the dyes. In the Ziehl-Neelsen method, heat is applied to drive the stain into the cell. The Modified Kinyoun method uses a wetting agent, Tergitol 7, for the same purpose. Once the cell is stained, acid-alcohol will not remove the light pink (acid-fast) color from the stained cell. The background is stained with methylene blue and serves as a color contrast for the pink tubercle bacilli.

11-24. *Culture.* You can culture the sediment on a variety of media, but Lowenstein-Jensen, Petragnani's, and Middlebrook 7H10 agar are commonly used. The first two are egg-base media which require a prolonged incubation period (up to 8 weeks). The Middlebrook medium is solidified with agar and grows tubercle bacilli more rapidly (about 3 weeks). The major drawback in its use is the need for a 2- to 5-percent CO_2 atmosphere. However, a CO_2 incubator, candle jar, or a culture of *Mycobacterium phlei* sealed in a plastic bag with the cultures will provide the correct proportion of gases.

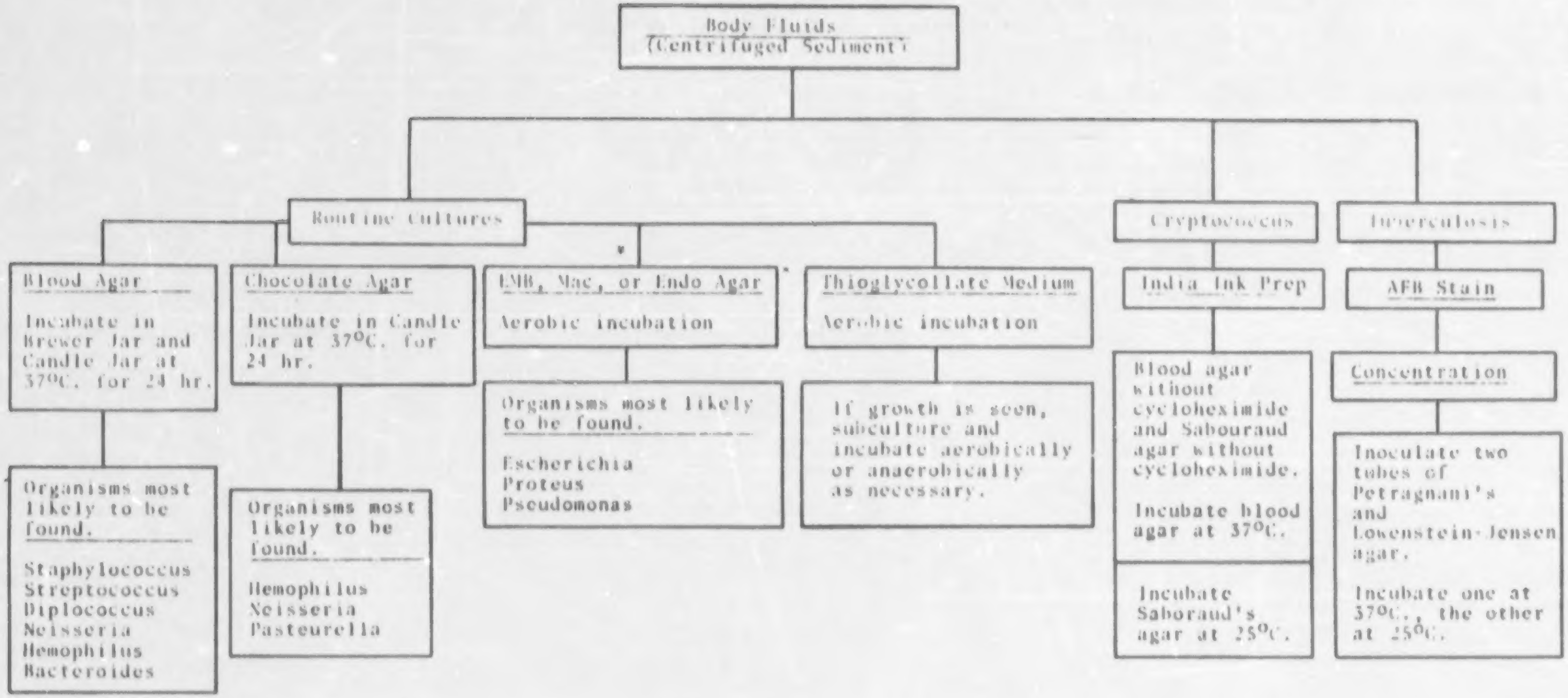
11-25. *Animal inoculation.* Animal inoculation is a valuable diagnostic tool to detect tubercle bacilli when only a few organisms are present in a specimen. The test also gives an indication of virulence. Inject cultures or concentrated clinical specimens subcutaneously or intraperitoneally into guinea pigs. The acid-fast organisms will develop a progressive and usually fatal disease. The lesions can be seen in any area of the body; however, invasion of the deep tissues suggests a mycobacterium of high virulence.

11-26. **Body Fluids.** Specimens that you will be asked to culture include spinal, synovial, pleural, pericardial, and peritoneal fluids. These specimens are collected by the physician from the spinal column, joints, and cavities of the lungs, heart, and abdomen. Among the organisms which you may encounter frequently in these body fluids are the following genera: *Neisseria, Diplococcus, Streptococcus, Staphylococcus, Hemophilus,* and occasionally *Mycobacterium.* One of the fungi chiefly responsible for meningitis is *Cryptococcus neoformans* (torula).

11-27. Again, you must consider whether to use routine or acid-fast techniques, and this is usually determined by the physician's provisional diagnosis. Since clotting is a factor to be considered in specimens of this type, you should usually [illegible] sterile heparin to prevent clotting while processing is underway. If the volume of fluid is small, you can culture the entire specimen. Otherwise, you should centrifuge the fluid and examine and inoculate the sediment to media according to the scheme shown in figure 35.

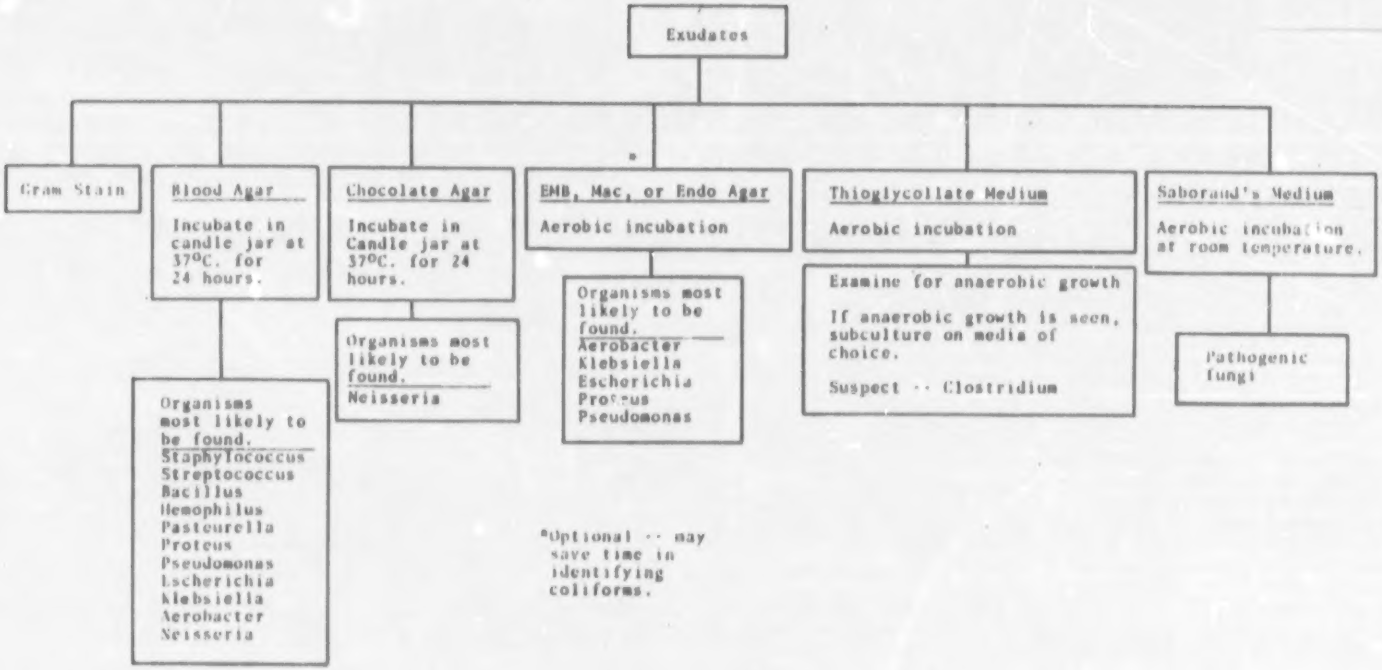
11-28. From the origin of the specimen in deep tissues, we can surmise that microbes found in body fluids are using an anaerobic form of respiration. Hence, the procedure for isolation differs slightly from that described for specimens from the respiratory tract. You should inoculate a portion of the specimen to two blood agar plates, one chocolate agar plate and one tube of thioglycollate medium. You can use an enteric medium as well if the direct smear of the sediment reveals Gram-negative rods. Incubate one blood agar plate and the chocolate agar plate under CO_2 tension in a candle jar. Incubate the second blood agar plate anaerobically in a Brewer jar for at least 24 hours at 37° C. The thioglycollate medium is incubated and subcultured as previously described. If acid-fast studies are indicated, you should inoculate TB media with an aliquot of the body fluid and prepare an acid-fast stain.

11-29. In addition to the usual Gram stain, do an India Ink preparation of the sediment to rule out infection with *Cryptococcus neoformans,* or one of the other pathogenic fungi. Mix a small drop of India Ink with a drop of the sediment on a microscope slide. If *C. neoformans* is present, a clear area—or capsule—will be outlined surrounding the individual or budding cell. In the case of a positive smear, the fungus media


Body Fluids
(Centrifuged Sediment)
Routine Cultures
Cryptococcus
Tuberculosis
Blood Agar
Incubate in Brewer Jar and Candle Jar at 37°C. for 24 hr.
Chocolate Agar
Incubate in Candle Jar at 37°C. for 24 hr.
*
EMB, Mac, or Endo Agar
Aerobic incubation
Thioglycollate Medium
Aerobic incubation
India Ink Prep
AFB Stain
Organisms most likely to be found.
Staphylococcus
Streptococcus
Diplococcus
Neisseria
Hemophilus
Bacteroides
Organisms most likely to be found.
Hemophilus
Neisseria
Pasteurella
Organisms most likely to be found.
Escherichia
Proteus
Pseudomonas
If growth is seen, subculture and incubate aerobically or anaerobically as necessary.
Blood agar without cycloheximide and Sabouraud agar without cycloheximide.
Incubate blood agar at 37°C.
Incubate Saboraud's agar at 25°C.
Concentration
Inoculate two tubes of Petragnani's and Lowenstein-Jensen agar.
Incubate one at 37°C., the other at 25°C.

*Optional -- may save time identifying coliforms.

Figure 35. Flowchart for processing body fluids.


Exudates
Gram Stain
Blood Agar
Incubate in candle jar at 37°C. for 24 hours.
Organisms most likely to be found.
Staphylococcus
Streptococcus
Bacillus
Hemophilus
Pasteurella
Proteus
Pseudomonas
Escherichia
Klebsiella
Aerobacter
Neisseria
Chocolate Agar
Incubate in Candle jar at 37°C. for 24 hours.
Organisms most likely to be found.
Neisseria
*
EMB, Mac, or Endo Agar
Aerobic incubation
Organisms most likely to be found.
Aerobacter
Klebsiella
Escherichia
Proteus
Pseudomonas
Thioglycollate Medium
Aerobic incubation
Examine for anaerobic growth
If anaerobic growth is seen, subculture on media of choice.
Suspect -- Clostridium
Saboraud's Medium
Aerobic incubation at room temperature.
Pathogenic fungi

*Optional -- may save time in identifying coliforms.

Figure 36. Flowchart for processing exudates.

listed in figure 35 should be inoculated with the specimen for incubation at both 37° C. and room temperature. Most fungi have an optimum temperature for growth lower than the 37° C. body temperature.

11-30. Cycloheximide (acidione) is an antibiotic commonly added to media to inhibit bacteria and saprophytic mold growth in fungus cultures. *Cryptococcus neoformans*, however, is one of the few pathogenic fungi sensitive to the additive. In figure 35 we have specified blood agar and Sabouraud's agar *without* cycloheximide; but if the stained smear suggests a pathogenic fungus other than *C. neoformans*, you should add the inhibitor to the isolation media.

11-31. **Exudates.** In Chapter 2 we defined an exudate as "material . . . passed through the walls of vessels into nearby tissues or areas of inflammation." Dorland[4] expresses it this way: ". . . any adventitious substance deposited in or on a tissue by a vital process or a disease." The bacteriologist usually pictures an exudate as fluid or semiliquid material collected from wounds, skin lesions, tissue abscesses, infections of the eye or ear, and similar pathologic conditions.

11-32. Most of the microbial pathogens known to man have been isolated at one time or another from exudates. Examples of this diversity were given in our earlier discussion of specimen collection and handling (Chapter 2, 3–13). As a matter of routine practice, therefore, culture media are selected which will support growth of most of the commonly found aerobes and anaerobes (fig. 36). The anatomical origin of the exudate, and the morphology of organisms seen in direct smears of the specimen often serve as a guide to the choice of isolation media.

11-33. For instance, Gram-positive rods found microscopically in a stained exudate from a deep wound would suggest infection with species of *Clostridium*, the anaerobes associated with gangrene and tetanus. In this case, you would emphasize anaerobic procedures by incubating a streak plate in a Brewer jar in addition to inoculating thioglycollate broth. Similarly, if an exudate reveals yeastlike forms or branching structures typical of fungi, we would watch closely for growth on Sabouraud's agar or one of the other media designed for isolating the pathogenic fungi.

11-34. **Urine Specimens.** The urinary tract can play host to a bacterial infection at a number of different points: the *kidney;* the *ureters*, which transport urine to the bladder; the *bladder*, itself; and the *urethral passage* leading to the external orifice. While one might expect the urinary tract to be free of microbes in a normal individual, samplings have shown that apparently healthy persons often void thousands of organisms per ml. of urine without clinical evidence of disease.

11-35. The finding of bacteria in "normal" urine has raised controversy as to the significance of a qualitative urine culture in detecting disease. Most physicians feel that the quantitative aspects are more significant than just the presence or absence of microorganisms, per se. Consequently, there is a trend toward reliance on colony counts as an aid to diagnosis.

11-36. It is now generally accepted that a bacteriuria (bacteria in the urine) of less than 10,000 organisms per ml. of a "clean catch" or "midstream" voided urine has little significance as an indicator of disease. Counts ranging from 10,000 to 100,000, however, suggest an infection; and colony counts larger than 100,000 per ml. indicate a disease state. Since bacteria can multiply in urine allowed to stand at room temperature, speedy processing of specimens is essential in performing colony counts. To demonstrate this point, let's assume that freshly voided urine contains only 1000 organisms per ml. With a generation time of about 20 minutes—not at all unusual among microorganisms—these 1000 cells can reproduce to a level of 130,000 per ml. within 2 hours and 20 minutes.

11-37. **Counting Techniques.** Of the several techniques available for counting bacteria in the urine, two will be described here:

- Dilution plating.
- Calibrated loop plating.

11-38. *Dilution plating.* In the first of these, an aliquot of the thoroughly mixed urine specimen is diluted 1:100 and 1:1000 in sterile saline. Then 0.1 ml. of the original specimen and the same volume of each of the two dilutions is transferred to the surface of separate blood agar plates for streaking with a loop needle. The number of colonies appearing after incubation, adjusted for the dilution factor, gives the bacterial content of the original urine specimen. Thus, if 400 colonies grow out of the *0.1 ml* undiluted aliquot, the urine contains 4000 organisms *per ml.* (10 × 400). You would expect, then, to find only about 4 colonies growing on the plate streaked with 0.1 ml. of the 1:100 dilution (4 × 10 × 100), and none on the 1:1000 dilution plate.

11-39. *Calibrated loop plating.* The second technique, calibrated loop plating, is more convenient in that it avoids the time-consuming act of preparing dilutions of the urine in saline. Wire loops are available which hold a measured vol-

---

[4] Dorland, W.A.N., *The American Illustrated Medical Dictionary*, 21st Edition, 1947, W.B. Saunders, Philadelphia & London, p. 533.

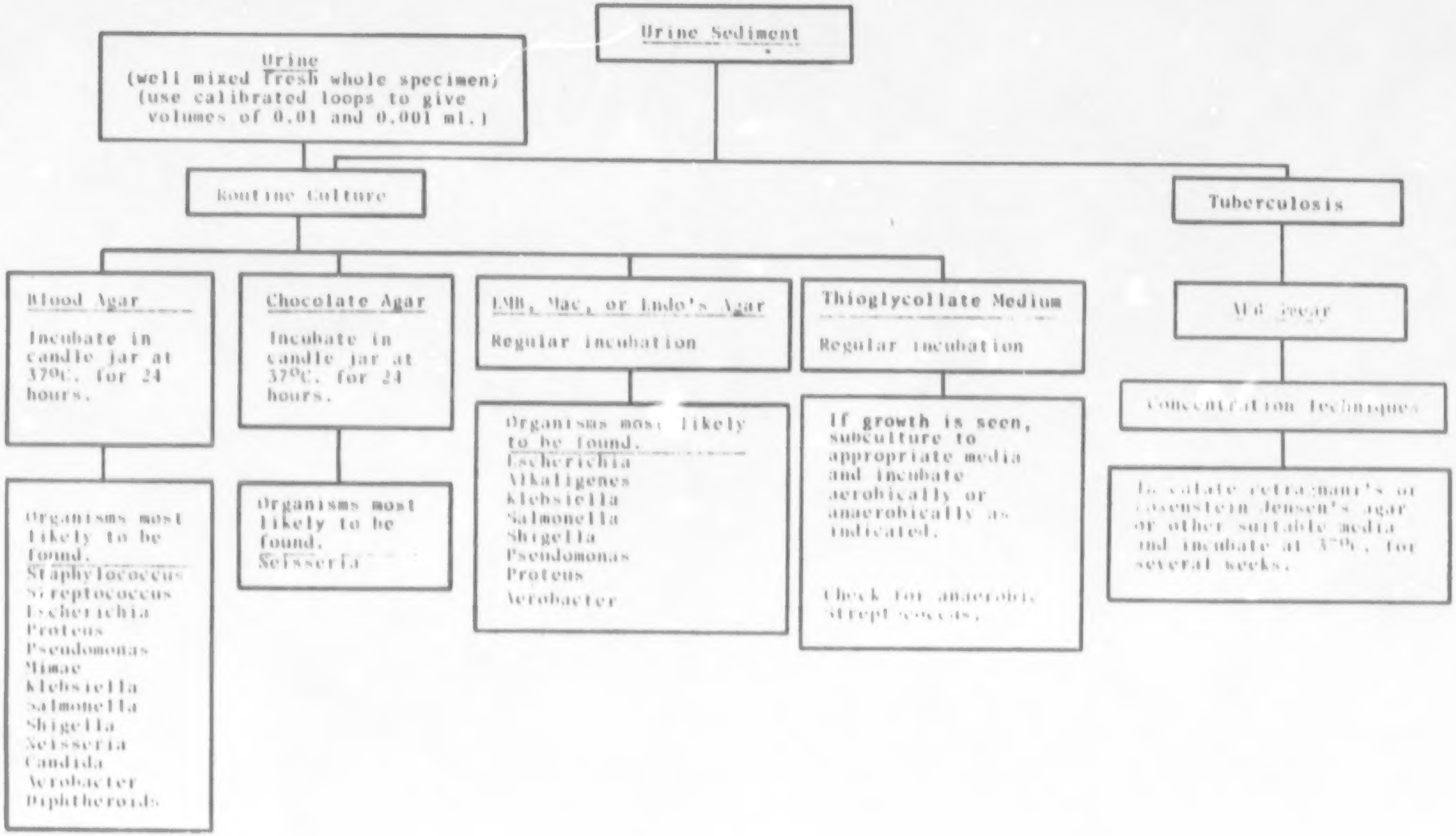

Urine Sediment
Urine
(well mixed fresh whole specimen)
(use calibrated loops to give volumes of 0.01 and 0.001 ml.)
Routine Culture
Tuberculosis
Blood Agar
Incubate in candle jar at 37°C. for 24 hours.
Chocolate Agar
Incubate in candle jar at 37°C. for 24 hours.
EMB, Mac, or Endo's Agar
Regular incubation
Thioglycollate Medium
Regular incubation
AFB Smear
Concentration Techniques
Organisms most likely to be found.
Staphylococcus
Streptococcus
Escherichia
Proteus
Pseudomonas
Mimae
Klebsiella
Salmonella
Shigella
Neisseria
Candida
Aerobacter
Diphtheroids
Organisms most likely to be found.
Neisseria
Organisms most likely to be found.
Escherichia
Alkaligenes
Klebsiella
Salmonella
Shigella
Pseudomonas
Proteus
Aerobacter
If growth is seen, subculture to appropriate media and incubate aerobically or anaerobically as indicated.
Check for anaerobic Streptococcus.
Inoculate Petragnani's or Lowenstein Jensen's agar or other suitable media and incubate at 37°C. for several weeks.

Figure 37. Flowchart for processing urines.

ume of urine, for example, 0.01 or 0.001 ml. A dip of the flame-sterilized loop into the specimen picks up a reasonably accurate volume of urine to be streaked on an agar surface. If you multiply the number of colonies by the dilution represented in the calibrated loop, the result gives you the number of bacteria present per ml. of specimen.

11-40. Since some of the microbes which cause urinary tract infections don't grow well, or at all, when surface-streaked to blood agar, it is usually a wise precaution to inoculate several other culture media (fig. 37) with the sediment from a centrifuged urine specimen. Although the urine might well contain a mixed population of organisms, a Gram stain of the sediment will often guide you in your choice of these supplementary media.

11-41. Urine specimens suspected of harboring the tubercle bacillus require a special concentration technique. First, a 24-hour pooled specimen is preferred. Start concentration by adding 2 or 3 grams of tannic acid to the pooled urine, shaking the sample thoroughly and letting the mixture stand overnight in the refrigerator. You can further concentrate the brown precipitate which forms if you decant the supernatant liquid and pack down the precipitate by centrifugation. This packed sediment is ready for inoculation to the TB media listed in figure 37, for staining, and for guinea pig inoculation.

11-42. **Fecal Specimens.** The human intestinal tract normally swarms with billions of microorganisms. It has been estimated in this regard that well over half of the dry weight of the stool consists of bacteria—both viable and dead cells. While some of the organisms found at any one time in the intestine are transient, having been ingested with food and water, many species must be considered normal flora. Of these latter species, perhaps *Escherichia coli* is the best known, since it is universally accepted as an indicator of fecal pollution in testing the quality of water and food.

11-43. The complex, interrelated flora of the intestines comprise representatives of many Gram-positive and Gram-negative genera. Certain of the Gram-positive forms are capable of causing disease, as in the case of staphylococcal food poisoning; but our foremost concern in fecal cultures is with the Gram-negative microbes which give rise to some of mankind's most prevalent and debilitating infections. We have reference here to the enteric diseases: typhoid and paratyphoid fevers, bacillary dysentery, food infections, and a variety of other gastrointestinal disturbances.

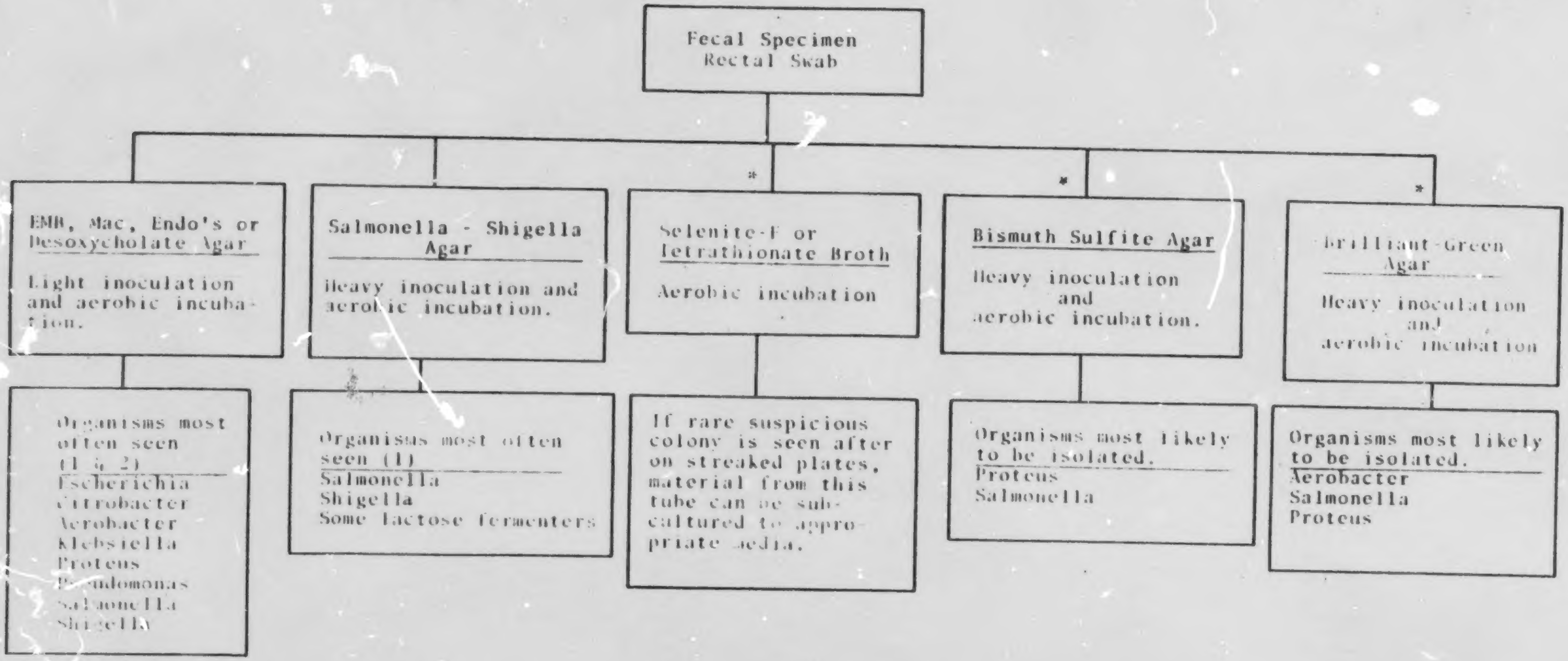
11-44. As a bacteriologist, your job is to carry out a series of isolation procedures which will successfully sort out the pathogens from the morphologically identical and taxonomically related saprophytes whose numbers predominate. Success calls for ingenuity in your choice and use of assorted enrichment, selective, and inhibitory media. Our procedural steps generally take the following sequence:

(1) Enrichment.
(2) Primary plating.
(3) Purification plating.

11-45. *Enrichment.* Enrichment is a device to increase the relative number of pathogens at the expense of the enteric saprophytes so that the pathogens will not be overgrown on a primary plating medium. Enrichment consists of inoculating either tetrathionate broth or selenite broth, or both, with 2 to 3 grams of feces or 2 to 3 ml. of a stool suspension in saline. After 18 to 24 hours of incubation at 37° C., subculture the broth to one or more of the plating media shown in figure 38 in order to obtain isolated colonies for study. Tetrathionate broth is somewhat toxic to *Shigella* species, so it is wise to set up a selenite enrichment tube along with the tetrathionate.

11-46. *Primary plating.* The choice of a primary plating medium depends to some extent on what organism the physician suspects of causing the disease in his patient. As a matter of routine, however, it is best to inoculate several of the available plating media in order to increase the probability of a positive isolation. These media differ sufficiently in nutrient constituents, selectivity, and inhibitory effects so that no one of them can be said to serve as a completely satisfactory medium for all enterics.

11-47. The media listed at the extreme left in figure 38, eosin-methylene blue (EMB), MacConkey's (Mac), Endo and desoxycholate agar permit growth of most enteric organisms. They also distinguish between nonlactose fermenters (most pathogens) and the usually harmless lactose fermenters. Color differences in the colonies permit you to spot the pathogens. Salmonella-shigella (S-S) agar, on the other hand, is actively selective for growth of the two genera from which the name of the medium is derived. It slows down reproduction of the saprophytic coliforms and allows pathogenic Gram-negative species to emerge. An added feature of S-S agar, beyond its selectivity, is the color distinction in colonies of pathogen and nonpathogen based on ability to ferment lactose. Bismuth sulfite agar is highly inhibitory to all enterics except the typhoid bacillus and certain of the other


Fecal Specimen
Rectal Swab
EMB, Mac, Endo's or Desoxycholate Agar
Light inoculation and aerobic incubation.
Salmonella - Shigella Agar
Heavy inoculation and aerobic incubation.
*
Selenite-F or Tetrathionate Broth
Aerobic incubation
*
Bismuth Sulfite Agar
Heavy inoculation and aerobic incubation.
*
Brilliant-Green Agar
Heavy inoculation and aerobic incubation
Organisms most often seen (1 & 2)
Escherichia
Citrobacter
Aerobacter
Klebsiella
Proteus
Pseudomonas
Salmonella
Shigella
Organisms most often seen (1)
Salmonella
Shigella
Some lactose fermenters
If rare suspicious colony is seen after on streaked plates, material from this tube can be subcultured to appropriate media.
Organisms most likely to be isolated.
Proteus
Salmonella
Organisms most likely to be isolated.
Aerobacter
Salmonella
Proteus

*Optional -- Selenite F or tetrathionate broth can be used as a holding medium or to increase number of pathogens.

Bismuth Sulfite and Brilliant Green Agars are used to aid in quick identification enterics.

(1) Organisms may be rapid, delayed or nonlactose fermenters.

(2) If organism is isolated resembling Streptococcus spp., inoculate SF broth and check for enterococci, Streptococcus faecalis.

Figure 38. Flowchart for processing fecal specimens.

Salmonella species. Due to this inhibitary action, the plating medium is regularly used for isolating *Salmonella typhi (S. typhosa)* when typhoid bacteria are sought in water, stools, or food.

11-48. Since primary plating media support the growth of most enteric disease producers, as a technician, you will often streak one or more of these media directly from the stool specimen at the same time you inoculate the enrichment broth. If you get a positive isolation on a streaked plate, you will have saved 18 to 24 hours in identifying the causative organism.

11-49. *Purification plating.* In any event, colonies of suspected pathogens (nonlactose fermenters) on primary plating media are usually picked and restreaked to MacConkey's, EMB, or one of the other formulations listed in figure 38 to provide a source of pure cultures for study on differential media. In some cases, of course, you can circumvent this purification plating step if well-isolated colonies of pathogens appear on the primary plating medium. The bacteriologist can then proceed directly to the inoculation of tubed differential agar, such as Kligler's or triple sugar iron (TSI) agar, to expedite species identification.

11-50. Much of our discussion of stool cultures has focused on means of separating lactose-positive (fermenters) from lactose-negative (nonfermenters) microbes among the Gram-negative genera. Those which utilize lactose as a source of energy, e.g., *E. coli*, can always be found in stool specimens. They hold little clinical significance except in occasional outbreaks of infant diarrhea.

11-51. The intestinal Gram-positive cocci are sometimes of interest to the physician, and these forms can be grown out on blood agar plates. On blood agar, however, you often encounter overgrowth by Gram-negative enterics, and purification plating to a selective medium becomes necessary. Fecal streptococci can be subcultured to *Streptococcus faecalis* medium (SF) which contains sodium azide to inhibit Gram-negative enterics. Similarly, mannitol salt agar (MSA) will permit growth in isolated colonies of Staphylococci to the exclusion of most other organisms.

11-52. **Blood Specimens.** In some microbial infections a *bacteremia* occurs as one phase of the disease process. "Bacteremia" simply means that bacteria are present in the blood stream. *Septicemia* is a term used to characterize a bacteremia with serious pathological manifestations—a so-called blood poisoning caused by pathogenic microorganisms and their toxic products. Regardless of whether organisms circulating in the blood entered from the intestine, a skin lesion, the lungs, the urinary tract, or some other site, the bacteriologist can aid in diagnosis by culturing a blood sample and identifying the microorganisms he finds there.

11-53. *Blood cultures.* Since blood, used in conjunction with other nutrients, furnishes an ideal growth environment for some of our most fastidious pathogens, it is not surprising that blood cultures are widely used, and with a great deal of success. It would be impractical to present even a partial list of the infectious disease agents which have been isolated from the bloodstream. With few important exceptions, any bacterium which has been propagated in the laboratory can be isolated by blood culture techniques. It is worth remembering, however, that a bacteremia is apt to be transient or intermittent, or to occur only during one well-defined phase of a given disease. For this reason, successive blood cultures over a period of time are usually indicated before the existence of a bacteremia can be ruled out.

11-54. In conventional practice, you use the blood obtained from a patient by venipuncture as part of the medium to grow out bacteria which might be present in that specific blood sample. One of the several available meat infusion or peptone broths forms the major component of the medium. Add the blood in 5 percent concentration. At a higher concentration the natural bactericidal properties of fresh blood would prevent growth of many of the pathogenic species.

11-55. The Castaneda technique of blood culture employs a rectangular bottle containing a solid slant of nutrient medium in addition to broth. Organisms in the blood adhere to the agar slant, making it relatively easy to pick colonies for staining. By contrast, in the aforementioned conventional broth culture technique, you must subculture organisms dispersed throughout the fluid medium to streak plates to obtain isolated colonies. This obviously involves more time before you can establish identity.

11-56. *Steps in processing a blood specimen.* In figure 39 we have outlined the steps in processing a blood specimen for culture. Draw sufficient blood for two culture bottles, one for aerobic cultivation at 37° C. and the other for anaerobic incubation under increased CO_2 tension. Examine the bottles visually each day for 21 days for evidence of growth. Negative cultures tend to remain clear during incubation. As our chart shows, a Gram stain of the fluid in positive culture bottles will indicate the subculture conditions and media you must set up to establish identity.

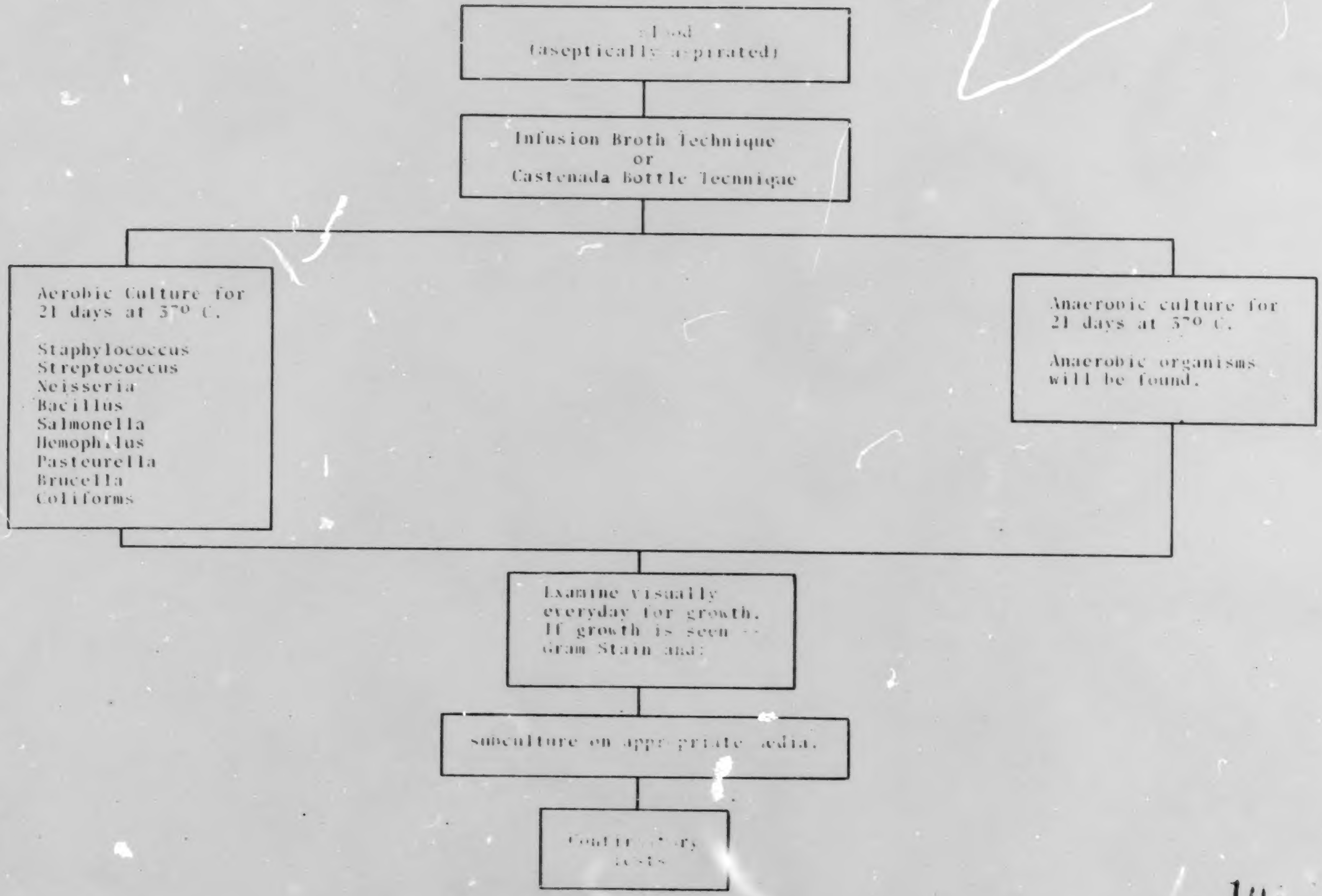

Blood
(aseptically aspirated)
Infusion Broth Technique
or
Castenada Bottle Technique
Aerobic Culture for
21 days at 37° C.
Staphylococcus
Streptococcus
Neisseria
Bacillus
Salmonella
Hemophilus
Pasteurella
Brucella
Coliforms
Anaerobic culture for
21 days at 37° C.
Anaerobic organisms
will be found.
Examine visually
everyday for growth.
If growth is seen --
Gram Stain and:
subculture on appropriate media.
Confirmatory
tests

Figure 39. Flowchart for processing blood specimens.

11-57. There are one or two other points to remember in performing blood cultures. Ideally, the specimen should be drawn before the physician begins antibiotic therapy. If therapy has already been started, however, you can sometimes overcome the bacteriostatic effect of drugs carried over to the medium in the blood sample. By limiting the blood inoculum to 5 percent of the total volume of culture fluid, the antibiotic is thereby diluted to a concentration that should fall below the inhibitory level. You can counteract sulfonamides in the blood specimen by incorporating para-aminobenzoic acid (PABA) (5 mg.%) in the culture broth. In addition, you can introduce the enzyme penicillinase into the culture to neutralize penicillin and streptomycin in the blood inoculum. For antibiotics which have no specific antidotes, you must rely on the dilution factor and the natural breakdown of the drug during the prolonged blood culture incubation period.

11-58. **Genitourinary Secretions.** There are a number of bacterial genera which give rise to infections of the lower urinary tract and the urethra. These infections are sometimes revealed by secretions from the external genitalia. Aside from the gonococcus which causes the venereal disease, gonorrhea, or specific urethritis as it is often labeled, enteric organisms can find their way into the gentiourinary passages and incite a pathological response. Moreover, microbes that are usually considered normal flora on and within the genitals occasionally become pathogenic when their environment is altered, as by a change in the state of the host's mucous membranes. Such infectious processes are sometimes referred to as nonspecific urethritis.

11-59. Figure 40 suggests the cultural conditions you should provide in attempting to isolate bacteria form genitourinary secretions. A stained slide prepared from the specimen is helpful in guiding you to a choice of media, although the slide often discloses a mixed population of microbial cells. The presence of Gram-negative intracellular diplococci, i.e., within the host's leukocytes, is usually diagnostic of gonorrhea. Thioglycollate medium and one or more enteric media serve to grow out the intestinal tract inhabitants and anaerobes which might be associated with the disease process. Blood agar and chocolate agar under CO_2 tension will promote growth of the various genera listed in figure 40.

11-60. The specimen is normally collected on a swab. Swabbed secretions from female patients are always obtained by the physician, but male patients may be sent to the laboratory for collection of a specimen. In this case the technician touches a sterile cotton swab to the secretion expressed by the patient from his penis. Inoculation of culture media and slide preparation follows. Prompt inoculation is indicated because many of the organisms found in genitourinary infections are especially susceptible to drying.

11-61. **Tissue Specimens.** On occasion the bacteriologist, will be asked to culture tissue, usually in association with submission of the specimen for histopathological examination. Specimens are obtained during surgical biopsy or autopsy procedures; they may consist of fluid as well as a solid tissue component.

11-62. Culture the liquid according to the processing scheme for body fluids (fig. 35). Ordinarily, you plant a fragment of the solid tissue in a liquid medium that supports growth of both aerobes and anaerobes. In this regard, thioglycollate medium is ideal as a general-purpose medium. In many cases the pathologist's guidance is valuable in selection of culture conditions. That is, a lung lesion may be typical of tuberculosis, or a liver nodule found at autopsy, may appear characteristic of one or another of the systemic bacterial infections.

11-63. A stained slide prepared from the fluid specimen, or an impression smear from solid tissue frequently points to the choice of media to be inoculated. The Gram stain reaction, the cell shape, and the presence or absence of unusual morphological features are especially useful in this respect.

12. Stain Technology

12-1. It is almost axiomatic that microscopic examination of a stained slide is the first task we undertake in identifying bacteria. Staining is necessary because most of the smaller internal and external structures of the cell are otherwise invisible. Indeed, certain of these structures become visible only after stains are applied in conjunction with intense heat or strong chemicals.

12-2. It is not surprising, then, that most staining procedures prove lethal to bacteria. So-called vital or supravital dyes are available to stain cells internally without destroying life functions, but these dyes have only limited usefulness in routine work. The staining techniques you most frequently use begin with a fixation process that quickly kills all cells on the slide. Fixation not only makes the cells adhere to the slide during staining, but it also reduces the hazard of handling exposed smears of living pathogenic organisms.

12-3. There are a few circumstances which require you to observe bacteria in the living state. The circumstance most often encountered in clinical laboratory practice is detection of

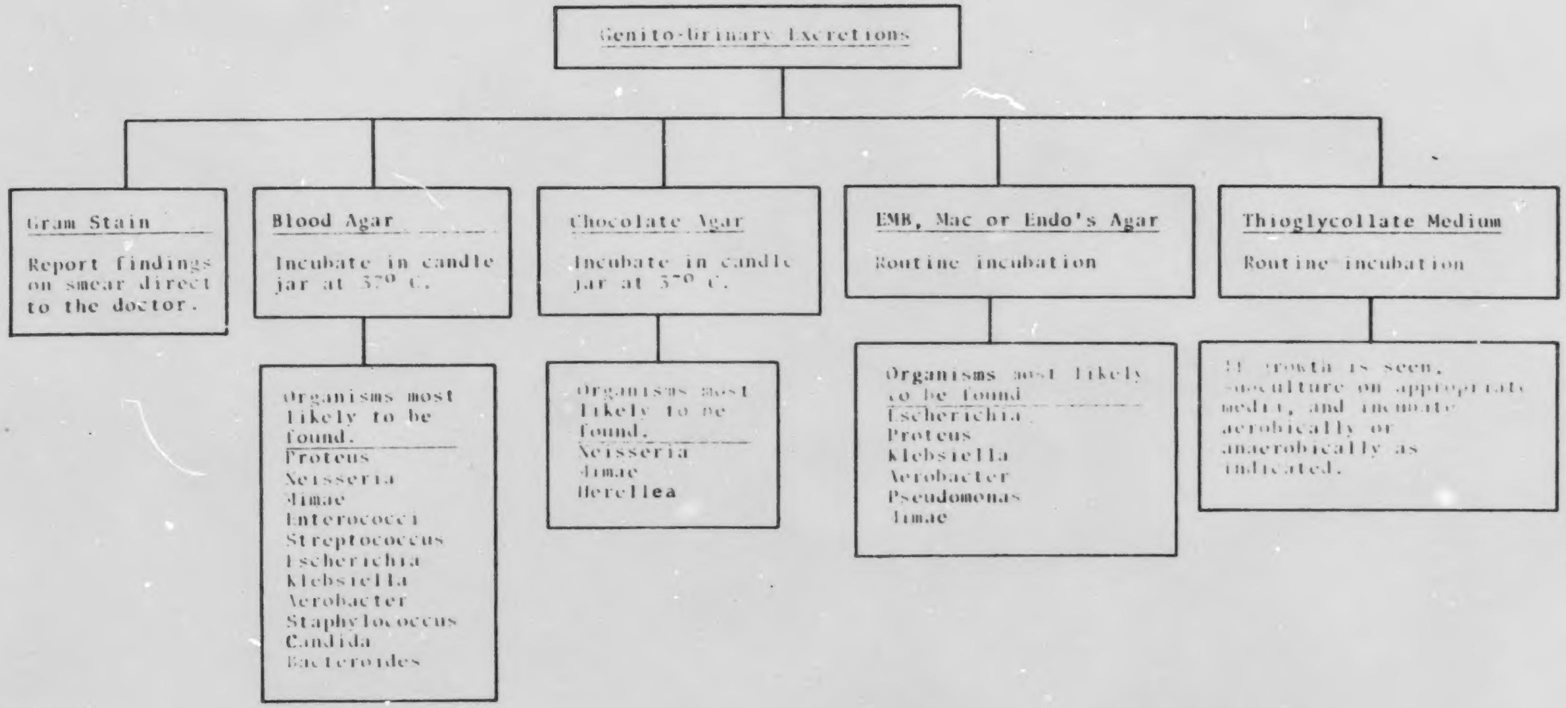

Genito-Urinary Excretions
Gram Stain
Report findings on smear direct to the doctor.
Blood Agar
Incubate in candle jar at 37° C.
Organisms most likely to be found.
Proteus
Neisseria
Mimae
Enterococci
Streptococcus
Escherichia
Klebsiella
Aerobacter
Staphylococcus
Candida
Bacteroides
Chocolate Agar
Incubate in candle jar at 37° C.
Organisms most likely to be found.
Neisseria
Mimae
Herellea
EMB, Mac or Endo's Agar
Routine incubation
Organisms most likely to be found
Escherichia
Proteus
Klebsiella
Aerobacter
Pseudomonas
Mimae
Thioglycollate Medium
Routine incubation
If growth is seen, subculture on appropriate media, and incubate aerobically or anaerobically as indicated.

Figure 40. Flowchart for processing genitourinary secretions.

motility. Later in this section, we will describe the wet mount or hanging drop technique of studying unstained bacteria. But, first, we must review some precautions in the proper preparation of smears, discuss certain fundamentals of stain technology, and explain some of the routine and special staining procedures performed daily by the bacteriologist.

12-4. **Smear Preparation.** There are three important points to keep in mind when you prepare smears for staining:

(1) Always use clean slides.
(2) Make more than one slide of a specimen.
(3) Air dry the smear completely.

It is essential to use only clean, unmarred glass slides because oily deposits, scratches, and residues of previous smears lower the quality of the stained slide.

12-5. You should generally make two or more smears from the same specimen for a number of reasons: the first slide might stain imperfectly; a special stain could be called for after initial microscopic observation; and multiple smears give a more representative picture of the bacteria present in the specimen.

12-6. *Air drying.* Complete air drying of the smear lays the groundwork for a uniform stain. It is always tempting to hasten the drying process by heating the wet slide with a Bunsen burner, but the risk of "cooking" the smeared cells so that they do not stain typically dictates against the application of a flame. It is permissible when time is short to place the wet slide on the laboratory bench near the base of the Bunsen burner so that heat radiating downward from the flame will accelerate drying. An alternative is to place the slide on a warm microscope lamp, but determine beforehand that the lamp is merely warm and not hot to the touch.

12-7. *Transferring specimen.* There are also several things to remember in transferring a specimen to the slide. Pus and serous exudates take the stain better if you spread them as a thin film over a large area of the slide. Moreover, masses of leukocytes and red cells are apt to obscure any bacteria present if the smear is too thick. For smears of sputum or feces, select small flecks of mucus or blood tinged particles. These elements are more likely to yield organisms on microscopic examination. You must first emulsify swabs containing dried material or meager specimens in a drop of sterile saline on the slide before spreading the film.

12-8. We have already noted that specimens of urine, spinal fluid, and other body fluids yield positive results more often if sediments are collected from the liquids by centrifugation. Transfer some of the sediment to a slide, using a loop needle or a clean, wooden applicator stick. Spread the sediment to make an even film. In working with sediment and other specimens (except those in which blood and tissue cells present a complicating factor), make a thicker smear as a rule than you ordinarily would in staining bacterial cells from broth or solid media. The concentration of microbes in clinical material is generally much lower than in laboratory cultures.

12-9. *Preparing liquid smears.* Smears of liquid (broth) cultures are prepared by depositing a loopful of the medium on a glass slide. If the broth is highly turbid, spread the droplet over an area slightly smaller than the size of a dime. When growth is scant, you may find it necessary to use 2 or 3 loops of broth to prepare a concentrated film. Bacterial growth on solid media presents a different problem. Pick a minute amount of material from a pure colony and emulsify it in a drop of fresh (bacteria-free) water on a glass slide. Picking is best done with a straight inoculating needle. The suspension of organisms should be only slightly cloudy, otherwise the smear will be too thick for microscopic observation of individual cells.

12-10. *Heat-fixing.* After you have made smears and allowed the slides to dry in the air, you must "fix" them by passing the slide through a Bunsen burner flame, smear side up, two or three times. The slide will feel slightly warm but not hot when you touch it to the back of your hand. If the slide gets too hot, the bacteria will be charred and altered in morphology. Heat also tends to modify the pH of the cellular material, a change that will consequently affect staining properties. Heat-fixing causes the smear to adhere so it will not be washed off the slide during the staining process. When the slide has cooled, the smear is ready for staining. If a number of routine cultures are to be examined using the same kind of stain, it is a practical idea to prepare several smears on a single slide.

12-11. **Staining Procedures.** Bacteria are colorless and transparent in their natural state. For the most part, fine structures are not visible unless you accentuate them by imparting a color to the cell. The staining process serves a dual purpose. The dye in the stain provides a visual contrast between various components of the cell, or between the cell and its background; secondly, the dye coating expands the dimensions of the smaller structures and thus improves the resolution of the light microscope.

12-12. *Description.* From the chemical standpoint a *dye* is an organic compound, usually structured of benzene rings and side groups which are specific for each dye. One such chemical group, the *chromophore*, gives the dye molecule its characteristic color. If the chromophore is an anion (negatively charged) in solution, the dye is said to be an acid dye. Conversely, a cationic chromophore (positively charged ion) denotes a basic dye. Eosin, one of the dyes incorporated in the familiar EMB agar for coliforms, is typical of the acid dyes. Methylene blue, crystal violet, basic fuchsin, and safranin are commonly used basic dyes.

12-13. In addition to the application of a *dye* to the smeared specimen, other reagents are usually employed as adjuncts in the staining process. A *mordant* is a substance that fixes a dye to the cell and thereby prevents the dye from being removed by decolorizers. The iodine reagent in the Gram stain is a prime example of a mordant. Iodine combines with the crystal violet to prevent washing out of the dye during decolorization of Gram-positive organism with acetone-alcohol solution. A *decolorizer*, in this instance acetone-alcohol, is a chemical solvent used deliberately to remove a dye from the material being stained. A *counterstain* refers to a secondary dye which follows in sequence the application of (or removal of) the principal dye in a staining procedure. Counterstains are useful in developing a color contrast, as in highlighting special structural features of a cell.

12-14. *Categories of stains.* For the sake of convenience, staining procedures can be classified into these four different categories:

(1) Direct stains.
(2) Indirect stains.
(3) Differential stains.
(4) Selective stains.

In *direct* staining, a dye is applied to bacteria to bring into view the general characteristics of cell morphology, such as shape, size, and grouping. *Indirect* stains color the background so that the cells stand out in contrast, but the bacteria, themselves, do not take the dye. India ink preparations exemplify this type of staining. *Differential* stains are used to distinguish between morphologically similar organisms on the basis of the relative affinity of cells, or cellular components, for a given dye. The Gram stain, which we will discuss shortly, is perhaps the best known example of a differential bacteriological stain. "Selective" stains, as the term implies, bring out specific structural features such as flagella, capsules, or spores.

12-15. The exact mechanism of dye action is not fully understood in each case, but we do know that some dyes undergo a firm chemical union with cellular components. With other dyes the staining involves only a physical absorption or simple coating action at the cell surface. In general, the acid dyes combine with basic elements of the cytoplasm to give an evenly stained appearance. But many bacteria, and notably the *Pasteurella* species, exhibit "bipolar" staining because cellular constituents concentrated at both ends of the cell stain more intensely or accumulate dye to a greater extent than other areas. Basic dyes tend to combine with the nucleic acid components of the cell; but since the nuclear material in bacteria is diffused throughout the cell, the basic dyes demonstrate gross morphology as well as certain internal structures.

12-16. Reagents used to stain bacteria can be a source of error if not properly prepared or applied. The concentration of the dye, the concentration of bacteria, and the time during which the dye is in contact with the microorganisms determine what the final color pattern will be. You must check each set of stains for the proper timing sequence if you are to achieve optimum results. And, too, a knowledge of culture conditions will often aid you in interpreting staining reactions. As an example, old cultures, particularly broth cultures, contain dead, dying, or atypical cells which will not stain uniformly. Bacteria grown in a carbohydrate broth may not yield typical results, particularly if the carbohydrate has been fermented with the production of acid.

12-17. With the foregoing information of a general nature as background, we can touch on several of the more important differential and selective stains. Two of these, the Gram and acid-fast staining procedures, are performed several times daily in a busy medical laboratory. Capsule and spore stains, while not done routinely, illustrate the technically complex selective staining procedures that the bacteriologist is called upon to perform from time to time.

12-18. *Gram staining.* Most bacteria can be classified into one of two categories on the basis of the *Gram* staining reaction: Gram-positive or Gram-negative. If the cells of a culture retain a crystal violet dye (cells are purple or blue) after washing with alcohol or acetone-alcohol mixture, these bacteria are said to be Gram-positive. Cells which release crystal violet under treatment with decolorizers and subsequently stain red with a safranin counterstain are termed "Gram-negative." The knowledge of whether an organism is Gram-negative or positive is critically important because this information limits the number of genera which must be considered in identifying an unknown bacterium.

12-19. The mechanism of the Gram stain is not fully known. The property of being Gram-positive appears to be associated with a ribonucleic acid component of the cytoplasmic membrane. This region of the bacterial cell has an affinity for the crystal violet-mordant (iodine) complex formed during the staining procedure. Gram-negative organisms do not show this affinity. It is true that crystal violet and iodine penetrate both Gram-positive and Gram-negative cells, but these reagents only form a stable compound in the Gram-positive cells. Bacteria which lack specific cellular substances responsible for binding the crystal violet-mordant fail to retain the violet stain when decolorizers are applied. The Gram-negative forms are, therefore, stained red by the safranin counterstain which follows the decolorizing step.

12-20. Although most bacteria are clearly either Gram-positive or Gram-negative, there are some species that exhibit a definite tendency to display both positive and negative cells in a stained preparation. These organisms are called Gram-variable. But whether positive, negative, or variable, the Gram reaction is species specific when properly performed and interpreted.

12-21. The Gram staining procedure is not unduly sensitive to variations in technique, but there are certain precautions you must watch in order to get a good stain consistently. One of these is the age and condition of the specimen to be stained. Gram-positive organisms may become Gram-negative as a result of autolysis, aging, acidity of the culture medium, improper temperature of incubation, or the presence of toxic substances (drugs, metabolic wastes). For best results, you should prepare Gram stains on cultures 18 to 24 hours old. Use a known Gram-positive organism as a control.

12-22. The quality of the smear is equally important. If you prepare films unevenly or too thick, dense deposits of material will retain crystal violet upon decolorization regardless of the Gram reaction. Under these conditions, falsely Gram-positive clumps of bacteria will be seen in an otherwise Gram-negative smear. Smears should be completely dry before heat-fixing, or else protein carried over into the smear from culture media or specimens will be precipitated. The background of the smear will then be difficult to decolorize and may contain debris and misleading artifacts.

12-23. Decolorization of Gram-positive cells can result if you use an iodine (mordant) solution which has deteriorated. Gram's iodine solution will remain stable for long periods of time when protected from light by storage in a dark bottle. When the iodine solution fades in color from brown to light amber or yellow, it is no longer suitable for use. Overly enthusiastic treatment with the decolorizing solution also results in a false Gram-negative reaction. *Immediately* after the washings become clear during treatment of the smear with alcohol or acetone-alcohol mixture, you must rinse the slide with water to prevent overdecolorization.

12-24. *Acid-fast staining.* Members of the genus *Mycobacterium* and certain *Nocardia* spp. possess relatively large amounts of lipids, fatty acids, and waxes within their cell wall. Such substances withstand penetration and staining of the cytoplasm by ordinary methods. When these resistant forms are heated during exposure to a high concentration of a basic dye in phenol, staining is more readily accomplished. Phenol serves as a carrier for the dye in penetrating the lipid layer. Once stained, the organisms resist decolorization with acid-alcohol even upon prolonged exposure. They are thus said to be *acid-fast*. Although all bacteria may be stained in this manner, only the aforementioned organisms, with rare exceptions, will resist decolorization with the acid-alcohol solution. The tubercle bacillus in a stained smear will appear pink to red because of the absorption of the carbol-fuchsin. The nonacid-fast bacteria, having been decolorized by acid-alcohol, will show up as blue from the methylene blue counterstain.

12-25. You will routinely use the Ziehl-Neelsen or Modified Kinyoun techniques in examining specimens or cultures for the presence of acid-fast bacilli. In the Ziehl-Neelsen procedure, time and temperature of heating the smear are somewhat critical. Overheating diminishes the color of the organisms to a pink or brown or may even distort the shape of the cell. Tergitol is incorporated in the Modified Kinyoun to reduce the surface tension between the cell wall of acid-fast organisms and the carbol-fuchsin stain. This is the role played by heat in the Ziehl-Neelsen stain. The heatless tergitol method is more advantageous in that it is less cumbersome and requires less time.

12-26. *Selective staining.* Selective stains, as we defined them earlier, aid you in establishing the identity of an organism by highlighting structures that usually can't be observed clearly with conventional stains. Capsules and spores are typical of these specialized structures. The *capsule* may be demonstrated by either negative or positive staining. You will see capsules most often in young cultures 4 to 6 hours old. Negative staining provides a dark background (usually black) against which the unstained organism and capsule contrast. The capsule is visible only because of the space it occupies. The dye, nigrosin, or India ink are most often used, but not all

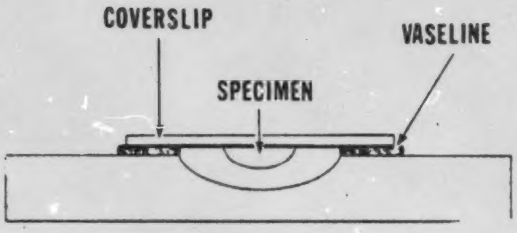


Figure 41. Hanging drop slide

brands of India ink are satisfactory for demonstrating capsules. Brands containing very finely divided carbon particles yield the best results. Inks should be free of bacterial contamination. They can be protected against contamination by adding phenol in 0.5 percent concentration by volume.

12-27. *Hiss stain.* The Hiss stain is useful in imparting color to the capsule, itself, i.e., positive staining. For best results, it is necessary to grow the test organism in a liquid medium containing serum or ascitic fluid for full development of the capsule. As an alternative you can mix blood serum and bacteria grown on solid media prior to air drying and heat fixing the slide. Bacterial capsules are composed of a polysaccharide which does not stain well by ordinary means. This capsule, however, can be caused to swell when mixed with normal serum. The serum allows the dye to penetrate. The combination of capsular swelling and dye impregnation is the basis of the Hiss stain. Use a copper sulfate solution instead of water to wash the stain from the slide, as water tends to act as a decolorizer.

12-28. *Spore staining.* Spores are developed in *Clostridium* and *Bacillus* species as a means of survival during unfavorable environmental conditions. In the laboratory, spores may form during routine culturing, and the presence of these bodies is a clear indication that you are dealing with one or the other of these genera. The bacterial spore is characterized by three distinct properties of interest to you in staining: (1) a high degree of heat resistance, (2) resistance to penetration by dyes, and (3) highly refractile when viewed microscopically. These properties account for the clear "hole" you see while examining spore formers stained with the Gram stain, methylene blue, or similar stains. The spores appear colorless with these stains, so in order to study a spore in detail, it is necessary to alter at least one of the three properties mentioned above. The Wirtz-Conklin spore stain is typical of the techniques available to you. Using this technique, you must steam the dye-covered smear for a brief period, utilizing the heat to drive the dye into the spore coat. Once the spore is stained, the dye is retained during rinsing, and the vegetative cell is then counterstained with a dye of contrasting color.

12-29. **Wet Unstained Preparations.** Occasionally, the microscopic examination of wet, unstained preparations can aid bacterial identification by revealing whether or not the organisms possess flagella. The most commonly used method is the hanging drop to detect motility of living bacteria in pure culture. The technique for preparation and microscopic observation of hanging drop mounts is illustrated in Figure 41. Deposit a drop of broth culture containing the cells on a coverslip and invert it over a depression slide. You can see flagellar motion as directional movement of individual cells. Do not confuse motility with Brownian movement which is a vibratory type of motion of the bacterial cells due to molecular bombardment. An alternate method is to use a plain glass slide and a vaseline "ringed" coverslip inverted over it. Although not as good as the hanging drop technique because of limited space between slide and coverslip, this method offers a quick way of examining bacterial growth when the concave depression slide is not available.

CHAPTER 3

Antimicrobial Agents, Drug Resistance, and Sensitivity Testing

IN CHAPTER 2 of this volume you studied some of the principles of disinfection and sterilization. You learned that microbes are susceptible to a wide variety of chemical compounds commonly referred to as "antiseptics" and disinfectants or "germicides." You also found that physical energy in the form of heat and radiation can be used to decontaminate materials in our environment which harbor pathogenic microorganisms.

2. But when we attempt to counter the pathogenicity of bacteria within the human body, the choice of antimicrobial agents is severely restricted. It is a biological fact that life processes in bacteria and man are so similar at the cellular level that most physical and chemical agents potent enough to destroy a bacterium inside the body are also toxic to the human host. It is not surprising, therefore, that until recent years few drugs (chemotherapeutic agents) were available to combat deep-seated infections in man safely and effectively.

3. With the discovery of a hitherto unrecognized class of biological substances, the antibiotics, an array of new therapeutic weapons became available to the physician. In this chapter we will show how representative antimicrobial agents act against bacteria, and examine some of the resistance mechanisms displayed by microorganisms. With that information as background, we will go on to discuss the laboratory procedures performed by the bacteriologist to assess the effectiveness of antibiotic therapy.

13. Antimicrobial Agents

13-1. For our purposes, the term "antimicrobial agent" embraces an assortment of therapeutic organic chemicals, including substances synthesized in the laboratory and those derived from living organisms. The widely prescribed sulfonamides typify drugs of the synthetic group. By common usage the term "antibiotic" is reserved for chemical substances produced by living forms—compounds like the penicillins—which in low concentration suppress the growth of microbes, or actually kill them outright. But since the underlying principle of sensitivity testing and assay is identical for most synthetic and naturally occuring chemotherapeutic agents, we will not emphasize differences in the source of the drug. We will exclude those antiseptic and germicidal compounds mentioned in Chapter 2 that are limited by toxicity to topical application or to disinfection of inanimate objects.

13-2. **Historical Review.** The history of man's search for antimicrobial substances is shrouded in antiquity. It has been said that the ancient Egyptians used a variety of medicinal plants which we now realize contain bacteriostatic or bactericidal constituents. A Roman Army surgeon, Pedanios Diiscorides, took advantage of his travels to compile information on the curative properties of plant extracts. His compilation, *Materia Medica*, was used as a textbook for centuries, and a successor compendium by the same title is still in use today.

13-3. Therapy of infectious disease remained in its infancy, however, until the investigations of Paul Ehrlich demonstrated in 1910 the selective toxicity of organic arsenicals for the spirochetes of syphilis. Ehrlich was convinced that the science of chemistry could provide a "magic bullet" that would destroy the pathogen without damaging the tissues of the human host. His successful studies opened the door to today's continuing search for new chemical substances combining the properties of low toxicity for humans and a high degree of bactericidal action against specific pathogens. Progress in discovering effective chemotherapeutic agents was slow, nonetheless. Not until 1935, when Domagk described the sulfonamides, was there available a potent antibacterial agent against the common Gram-positive and Gram-negative cocci and enteric organisms.

13-4. The era of the antibiotics as antimicrobial substances came even more slowly. Louis Pasteur in the 19th century reportedly observed inhibition of the anthrax bacillus in mixed culture with other bacteria, but it was Vuillemin in 1889 who introduced the term "antibiosis" to denote antagonistic interactions between living organisms. A milestone was reached in 1929 when Sir Alexander Fleming detected the lytic effect on staphylococci of a substance produced by a common mold, *Penicillium notatum*.

13-5. Several years later this substance was identified as crude penicillin, the forerunner of several closely related compounds which are now modified synthetically to enhance their lethality for bacteria. The discovery of penicillin prompted a systematic screening of soil bacteria, fungi, algae, and even the higher plants for other antibiotics. Examples of well-known successes are streptomycin (1944), chloramphenicol, and polymixin (1947), aureomycin (1948), and additional penicillins, tetracyclines, and erythromycin in the 1950s. The organic chemist also made several important contributions during the same period, as drugs such as p-aminosalicylic acid and isonicotinic acid hydrazide (isoniazid), useful in treating tuberculosis, were synthesized in the laboratory.[5]

13-6. **General Characteristics.** The search for new drugs continues, as science seeks substances active against viruses, pathogenic fungi, protozoan infections, and bacteria resistant to currently available antibiotics. Most of the natural antibacterial agents of low toxicity have been isolated from species of *Actinomyces* and *Streptomyces*, plus a few genera of the Fungi Imperfecti (asexual fungi), e.g., *Penicillium* and *Aspergillus*. Many other soil species produce antibiotics, however, and thousands of cultures have been screened in an effort to find products possessing selective toxicity for pathogens.

13-7. As one might judge from the variety of microorganisms that produce antibiotics, there is no single molecular configuration common to all antimicrobial agents. We might also expect, then, to find differences in the manner in which a given pathogen is affected by the various agents, and this turns out to be the case. If we group antimicrobial substances in terms of their effect on cell functions, five major categories can be recognized.[6] There are agents which disrupt:

- Cell wall formation.
- Cytoplasmic membrane function.
- Protein synthesis.
- Nucleic acid metabolism.
- Intermediary metabolism.

13-8. It will become apparent later in our discussion of the mechanism of action of representative drugs that two antibiotics may differ radically in the way they interfere with cellular activities at the molecular level. However, the net result of the interaction between agent and cell component can generally be seen in the interruption of one or more of the vital functions listed above.

13-9. Antimicrobial substances are usually characterized as either *bacteriostatic* or *bactericidal*, although some of them possess both bacteriostatic (inhibitory) and bactericidal (lethal) properties, depending upon drug concentration and the type of bacterial pathogen involved. This difference in effect is understandable if we examine the nature of the cell function that is altered. For instance, an agent is clearly bactericidal if its action so weakens the main supporting structure—the cell wall—that the high internal osmotic pressure of the cytoplasm causes lysis of the cell. On the other hand, a drug that upsets nucleic acid synthesis may prevent the cell from reproducing even though the functions of respiration and growth remain intact. The end result of this inhibitory, or bacteriostatic, effect is that multiplication of the pathogen is suppressed, and the natural defenses of the host are given an opportunity to overcome the infection.

13-10. The capacity to interfere in fundamental cell processes has given the antimicrobial agents a usefulness that extends beyond their primary value in treating disease. These agents are not only helpful in the laboratory, but they are economically important to industry as well.[7] They can be used:

- As selective inhibitors in culture media.
- In classifying bacteria taxonomically on the basis of drug susceptibility.
- In preventing contamination of tissue cultures.
- For preserving refrigerated meats and other foods.
- As food additives to stimulate growth of livestock for meat production.

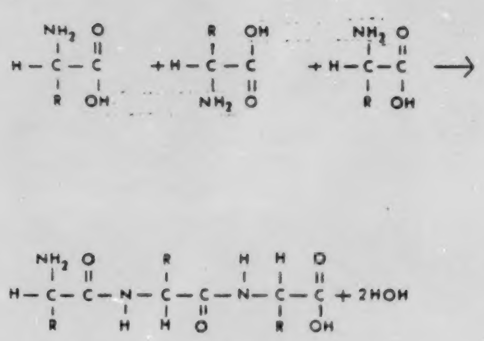
The stimulatory effect on weight gain in live-

---

[5] This short historical review was based on more extensive information given by Loyd W. Hedgecock in *Antimicrobial Agents*, No. 3 in Medical Technology Series, 1967, Lea and Febiger, Philadelphia, pp. 2–4, 138–40; and by Sir Graham Wilson and A. A. Miles in *Topley and Wilson's Principles of Bacteriology and Immunity*, fifth edition, Vol. 1, 1964, Williams and Wilkins Co., Baltimore, pp. 173–217.

[6] Hedgecock's development of this classification, the previously referenced *Antimicrobial Agents*, was based on a concept proposed by D.S. Feingold in *New England Journal of Medicine*, 269:900, 1963.

[7] Wilson and Miles, op. cit., p. 175.



Three amino acid molecules combine to form a polypeptide chain. "R" indicates the remainder of the amino acid molecule not shown in the drawing.

Figure 42. Illustration of peptide linkage.

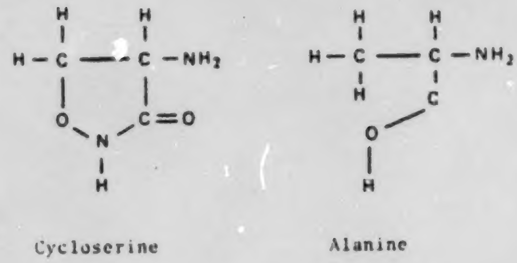


Figure 44. Structural formulas of cycloserine and alanine.

stock is not well understood, but the other uses stem directly from the same suppressive or lethal action displayed by the antimicrobial agents in combating disease in humans.

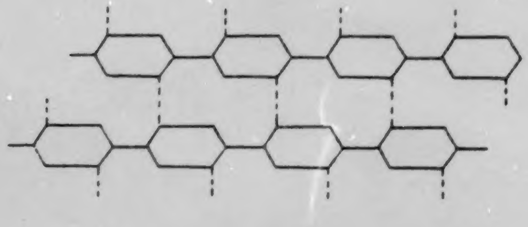
14. Mode of Drug Action

14-1. Beginning with the bacterial cell wall and proceeding deeper into the cell, we will follow the functional classification of antimicrobial agents set forth above to show how representative chemotherapeutic drugs react at the molecular level.

14-2. **The Cell Wall.** The bacterial cell wall is a complex structure containing various combinations of lipids, carbohydrates, and proteins. But chemical substances known as *mucopeptides* comprise the main building blocks from which the cell wall is constructed. Each building block is fashioned from long chains of a polysaccharide held in place by cross links formed by amino acids. A polysaccharide is a form of carbohydrate containing four or more molecules of the simple sugars or their derivatives.

14-3. You will recall that amino acids (the main constituents of protein) are carbon compounds having at least one free amino (NH_2) group and one or more carboxyl (COOH) groups. The amino acids that keep the polysaccharide chains in proper position are themselves bound together by a so-called "peptide linkage" in which the NH_2 group of one amino acid combines with the COOH group of another, as shown in figure 42. The union of two amino acids yields a peptide, and a molecule formed by several peptides is called a polypeptide. We can thus visualize the mucopeptide building block of the cell wall as a lattice-like structure in which parallel strands of polysaccharide are held in their respective positions by lateral bridges formed between the strands by the polypeptides. This structure is illustrated in figure 43.

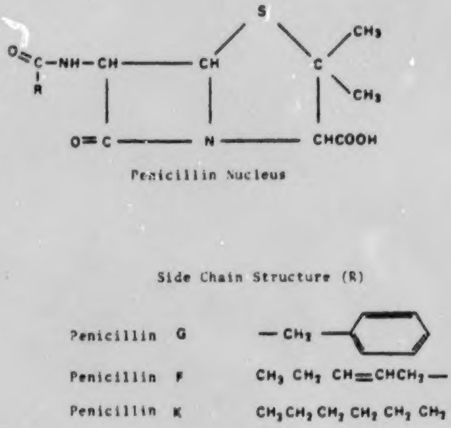
14-4. The rigid cell wall gives shape to the bacterium, protects the cell from mechanical injury, and provides structural support for the underlying cytoplasmic (protoplast) membrane which regulates the flow of nutrients into and out of the cell. Because of the great difference in the



Horizontal chains represent the polysaccharide component.

Vertical lines are formed by polypeptides.

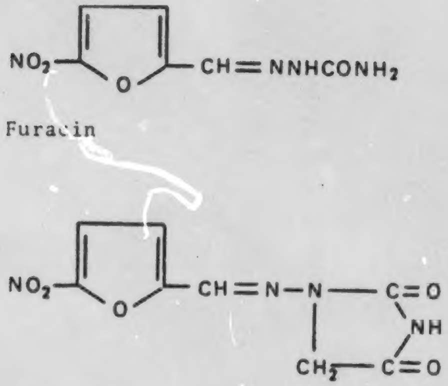
Figure 43. Sketch of mucopeptide unit.



Penicillin F: $CH_3\,CH_2\,CH{=}CHCH_2—$

Penicillin K: $CH_3CH_2CH_2CH_2CH_2CH_2CH_2—$

Figure 45. Structural formula of penicillins.



The side chains on the nitro furan ring nucleus give the compounds their antimicrobial specificity.

Figure 46. Structural formula of nitrofuran compounds.

concentration of solutes within the cell (as opposed to the external medium), the osmotic pressure inside the cytoplasmic membrane is high. Without an intact cell wall, the membrane cannot maintain the proper osmotic relationship for normal functioning. As we pointed out in our discussion of a bactericidal agent (paragraph 13-9), the pressure differential may also lead to lysis and death of the cell.

14-5. There are several antimicrobial agents that in one way or another adversely affect the cell wall. We will talk about bacitracin produced by *Bacillus licheniformis*, cycloserine *(Streptomyces spp.)*, the penicillins *(Penicillium spp.)*, and the synthetic nitrofuran compounds.

14-6. *Bacitracin.* This agent, a mixture of polypeptides, blocks the integration of mucopeptide units into the wall structure. We think that bacitracin interferes with the orderly linking of the polypeptide side chains that bridge the polysaccharide strands.

14-7. *Cycloserine (Oxamycin).* This antibiotic, active against many Gram-positive and Gram-negative microbes including the tubercle bacillus, is structurally similar to the amino acid, alanine. (See fig. 44.) When the antibiotic is present, the enzymes that control synthesis of the peptide component randomly select a molecule of cycloserine (a "competitive analog") instead of the natural alanine. The antibiotic, although sufficiently similar to alanine to engage the enzyme, cannot be fitted into the peptide portion of the building block. As a result, the mucopeptides function imperfectly because the sequence of amino acids within them is abnormal.

14-8. *Penicillins.* There are several different penicillins. The nucleus common to all of them is a cyclic dipeptide of cystine and valine. Study figure 45. Note that it is the side chain, attached to the nucleus at the point "R" (radical) in the diagram, that gives the various penicillins their distinctive actions against different groups of microorganisms. Several representative side chains are shown in this figure. The penicillins are thought to affect the bacterial cell at more than one site, although the exact nature of the reactions is still not clear. Laboratory experimentation has disclosed three main effects on susceptible organisms: a blocking of amino acid uptake from the external environment; inhibition of the synthesis of certain enzymes by the cell; and interference with cell wall formation. With regard to the cell wall, the effect is believed to be either an interruption of the process in which the mucopeptide building block is fabricated from its component parts, or disruption of the polypeptide cross links between the strands of polysaccharide in the mucopeptide.

14-9. *Synthetic nitrofuran compounds.* Among the bacteriostatic and bactericidal drugs synthesized in the chemical laboratory are the nitrofuran compounds. Figure 46 illustrates these compounds which are useful in treating urinary tract infections by Gram-positive and Gram-negative forms. As in the case of the penicillins, there seem to be multiple sites of drug action that is, intracellular enzyme systems, the cytoplasmic membrane, and the cell wall. Because mucopeptide precursors (raw materials) accumulate in culture media containing the nitrofurans, there is speculation that the drugs inhibit fabrication of the mucopeptide unit in its finished form.

14-10. **The Cytoplasmic Membrane.** A thin membrane located directly beneath the cell wall encases the bacterial protoplasm. This cytoplasmic or protoplast membrane provides a barrier against the indiscriminate flow of fluids and metabolites into and out of the cell and maintains the proper interior osmotic pressure for life functions. The structure houses the enzymes that mediate the selective transport of sugars, amino acids, and essential ions across the membrane into the cell. In addition, there is evidence that the cytoplasmic membrane participates in the synthesis of other structures, such as the cell wall and appendages, e.g., flagella.

14-11. More than half of the membrane is protein in nature, but lipids and carbohydrates are present in significant amounts and in chemically complex molecular arrangements. The ac-

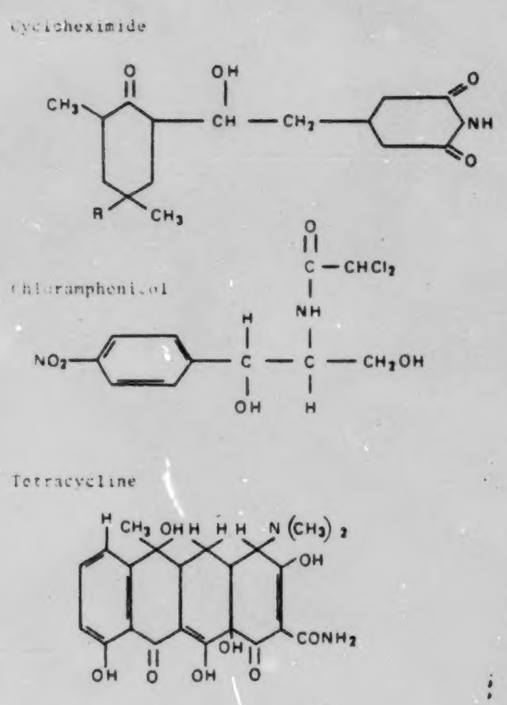


Figure 47. Compounds affecting protein synthesis.

tion of antimicrobial agents is generally manifested in one of two ways. First, combining of the agent with a vital component of the membrane can impair the transport of materials into or out of the cell. Secondly, the union of agent and membrane can result in a loss of selective permeability so that essential constituents "leach" out of the cell into the exterior environment.

14-12. Several antimicrobial agents active against the pathogenic fungi (but not against bacterial pathogens) owe their effect specifically to a joining of the agent with sterol components of the cytoplasmic membrane. Bacteria are resistant because they lack the sterols (cyclic alcohols) with which the antibiotics combine. Amphotericin B (*Streptomyces nodusus*), filipin (*Streptomyces filipinensis*), and nystatin (*Streptomyces noursei*) are members of this group of antifungal substances whose action disorganizes membrane permeability and permits vital intracellular elements to be lost.

14-13. Among the antibacterial drugs affecting the cytoplasmic membrane are the polypeptides polymyxin and tyrothricin. *Bacillus polymyxa* produces several polymyxins, each differing in amino acid content, but the basic molecular configuration is the same. The polymyxins apparently form a complex with phospholipids of the lipoprotein present in the cytoplasmic membrane of Gram-negative organisms. This union causes a breakdown in the membrane's capacity to regulate permeability, and cell constituents diffuse to the exterior.

14-14. Tyrothricin is a mixture of cyclic polypeptides, labeled gramicidins and tyrocidins, elaborated by *Bacillus brevis*. These antibiotics interfere with the energy-yielding phosphate reactions that are necessary to cell respiration. Beyond this, the polypeptide agents from *B. brevis* exhibit properties similar to the polymyxins in disrupting the normal control of permeability.

14-15. **Protein Synthesis.** Before we learn how antimicrobial drugs exert their effect on bacterial protein synthesis, we should review briefly the major steps in the manufacture of protein by the cell. These steps are carried out by ribonucleic acid (RNA) components acting under the genetic influence of deoxyribonucleic acid (DNA) found in the nuclear portion of the cell.

14-16. Actually, we are concerned here with three distinct forms of ribonucleic acid:

- Ribosomal RNA.
- Soluble RNA.
- Messenger RNA.

14-17. *Ribosomal RNA.* The preponderance of the RNA occurs in the *ribosomes*, which are free particles, or aggregates of particles (polyribosomes), that combine individual amino acids into peptides, the building blocks of the new protein. The ribosomes can be looked upon, then as the cell's manufacturing plant for protein.

14-18. *Soluble RNA (sRNA).* This constitutes 10 percent or less of the total RNA. Soluble RNA molecules select specific amino acids from the intracellular pool of these compounds and deliver them to the ribosomes for fabrication into protein. Soluble RNA thus performs a transport function between raw materials and the protein manufacturing site of the cell.

14-19. *Messenger RNA (mRNA).* Messenger RNA which comprises the remaining small fraction of the total RNA, can be pictured in the role of the plant production chief, since mRNA transmits from the nuclear DNA (the plant manager) to the ribosome instructions on the makeup of the new protein that is to be synthesized. This mRNA information, or genetic code is [illegible] by the ribosome as the latter links together in proper sequence the amino acid molecules supplied by sRNA.

14-20. The intricate process of protein synthesis, greatly simplified here for our purposes, can be disrupted at many points in the chain of events. First, there are antimicrobial agents that in therapeutic concentration cause destruction or gross impairment of major systems in the cell.

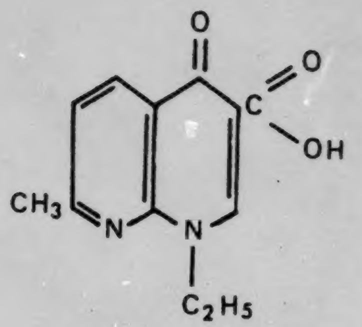


Figure 48. Structural formula for nalidixic acid.

For instance, exposure to streptomycin *(Streptomyces griseus)* results in a general breakdown of DNA, while ribosomes formed in the presence of chloramphenicol *(Streptomyces venezuelae)* are intact, but so structurally disorganized that the protein they synthesize is imperfect and nonfunctional.

14-21. Usually, however, you will find that the adverse effect of the agent is more subtle. It is apt to be focused on one of the reactions carried out by components of the sRNA—ribosome—mRNA complex as they actively produce new protein. In the next paragraph we will show some examples of postulated mechanisms that may account for the action of antimicrobial agents in protein synthesis. These assumptions are based on research conducted with a variety of experimental systems.

14-22. Lincomycin from *Streptomyces lincolnensis* is believed to interfere with the activation or transfer of amino acids from the pool of these raw materials. *Chloramphenicol* and the tetracyclines, as shown in figure 47, apparently block the release of amino acids from the carrier, sRNA, to the ribosome. Similarly, by combining with the ribosome at one specific point on that organ, erythromycin *(Streptomyces erythreus)* blocks the attachment of the incoming sRNA molecule and its amino acid. Streptomycin binds the ribosome at still another point to retard the attachment or movement of the mRNA molecule. In this instance the result is a misreading of the genetic code for the makeup of the protein that is manufactured on the ribosome. In the final stage of protein synthesis, cycloheximide *(Streptomyces griseus)*, also shown in figure 47, and puromycin *(Streptomyces alboniger)* in some way prevent the correct placement of amino acids in their respective positions in the developing peptide molecule.

14-23. **Nucleic Acid Metabolism.** Although many compounds have been discovered that upset functions in the nuclear elements of the microbial cell, few are suitable for therapeutic purposes. Nucleic acid metabolism in all species is so similar that an agent toxic to one form is generally poisonous to all. Little is known about the exact mode of action of drugs in this group, but we will mention two typical examples.

14-24. The actinomycins produced by *Streptomyces antibioticus* slow down RNA synthesis in the cell. We believe that the antibiotics bind the DNA of the nucleus at a location which obstructs the activity of RNA polymerases. These are the enzymes that put together RNA molecules in accordance with genetic instructions from the DNA. Nalidixic acid, shown in figure 48, is a synthetic drug useful in treating urinary tract infections by Gram-negative rods. The antimicrobial agent inhibits DNA synthesis which, in turn, retards the production of RNA and protein. The specific nature of the bactericidal reaction has not yet been determined.

14-25. **Intermediary Metabolism.** We can use this term in its broadest context to include the energy-yielding reactions taking place in the cell (respiration), the breakdown of complex nutrients to simplified usable form (catabolism) and the buildup of cell constituents from these simpler substances (anabolism). Para-amino salicylic acid (PAS), active only against the mycobacteria, and the sulfonamides, useful in treating infections caused by the cocci and the Gram-negative rod forms, exemplify drugs that derange metabolic patterns in the bacterial cell.

14-26. Both of the above drugs are involved in para-aminobenzoic acid (PABA) metabolism, although the inhibitory mechanism is probably not identical. PABA is an essential metabolite (required for growth) for several genera of organisms. In still others, it forms a part of the folic acid molecule. Folic acid is a growth factor associated with coenzymes that assist in transferring single carbon units.

14-27. In figure 49 note the structural resemblance of PABA, PAS, and the sulfonamides. We can look upon PAS and the sulfonamides

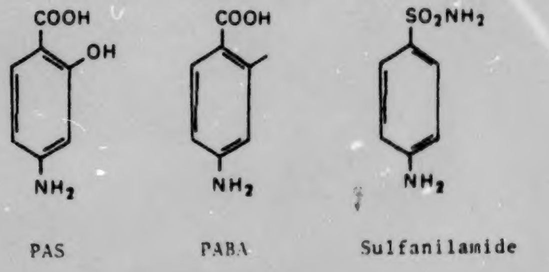


Figure 49. Structural formula for PAB, PAS, sulfanilamide.

as "pseudometabolites" or "competitive analogs" which the cell mistakenly builds into various molecular structures that require PABA for normal functioning. The result is a blocking of metabolic pathways when the faulty molecules enter into cellular reactions.

15. Mechanism of Antibiotic Resistance

15-1. Soon after penicillin came into wide usage in the 1940s, it was recognized that some strains of *Staphylococcus aureus* were developing resistance to drug concentrations that were formerly lethal. Resistance was attributed to production of an enzyme, "penicillinase," that degraded the penicillin molecule into harmless fragments. The full extent of the problem of drug resistance was not realized, however, until research in the 1950s and early 1960s uncovered the existence of resistance factors that are transmissible between microbes.

15-2. In 1955, an outbreak of Shigella infections in Japan yielded cultures that were resistant to four different drugs: chloramphenicol, tetracycline, streptomycin, and the sulfonamides. It has since been shown that drug tolerance can be transferred among several medically important species, including *Salmonella* and *Shigella* species, *Escherichia coli*, *Vibrio cholera*, and *Pasteurella pestis*. Resistance has been found in a single organism to as many as seven of the commonly employed antimicrobial agents.* It is becoming increasingly important, therefore, to understand the genetic and cellular mechanisms that account for enhanced tolerance to therapeutic agents.

15-3. **Genetic Basis of Resistance.** We can distinguish two, and possibly three, mechanisms whereby cells acquire drug resistance; but there are no indications that all of these are available to a given bacterial species. One postulated mechanism, a nongenetic variation or "physiological adaptation" will be discussed later in this section. The other two mechanisms, i.e., spontaneous mutation within a single cell and the exchange of genetic material between cells, have a genetic basis. We know that mutants arise naturally in bacterial cultures with a frequency that can be predicted accurately in many cases. For example, resistance to isoniazid reportedly occurs in approximately one cell of each million progeny of a single sensitive tubercle bacillus. It is now recognized that mutation to drug tolerance is a normal characteristic of the genes in many pathogenic bacterial species.

15-4. The exchange of genetic material between cells of the same or closely related species can take three forms:

(1) Transformation.
(2) Transduction
(3) Conjugation.

15-5. *Transformation.* Transformation is the uptake by one cell of nuclear elements (genes) derived from another cell. The newly incorporated factor for drug tolerance imparts a corresponding resistance to the recipient cell. Transformation is demonstrated experimentally by adding DNA extracted from resistant microbes to growing cultures of drug-susceptible cells. The extent to which this transfer mechanism may take place under natural circumstances has not been determined with certainty.

15-6. *Transduction.* This exchange is a transfer of genetic material mediated by bacteriophages, the virus particles that parasitize many species of bacteria. During infection of the bacterium with certain so-called lysogenic phages, the host's drug-resistance factor is incorporated into the nuclear structure of the replicating viruses. Upon release from the bacterial cell, these newly formed viruses invade other cells and deposit the genes that confer resistance.

15-7. *Conjugation.* The aforementioned outbreak of Shigellosis in Japan led to unsuspected findings on the sexual cycle in genera of the enteric organisms. In a fashion comparable to mating in higher forms of life, male and female strains undergo *conjugation*, a process in which genetic material is passed during direct cell contact. If one cell is drug resistant, this trait can be passed along to the partner. The recipient can in turn transfer the resistance characteristic (R) to another microorganism. The R factor is an aggregation of genes closely associated with—and dependent upon—another gene complex variously referred to as the "resistance transfer factor," the "fertility factor," or the "sex factor." This transfer factor bestows the ability to conjugate and to transfer the R factor during the period of physical contact between cells. Recent work gives evidence that the R factor can also be transmitted by bacteriophage.

15-8. **Cellular Mechanisms.** We don't know how possession of the R factor enables a bacterium to tolerate concentrations of an antimicrobial agent that would prove lethal to other members of the same species. Speculation centers on restrictive permeability of the cell wall to the drug, production of enzymes that inactivate the agent, resistant ribosomes, and other physiological defenses. We do know, however, that drug tolerance in many species is at least partly, and perhaps exclusively, under genetic control.

---

* A more complete report of this outbreak in Meynell, Elinor G., G. G. Meynell and Naomi Datta, "Phylogenetic Relationships of Drug-Resistance Factors and Other Transmissible Bacterial Plasmids," *Bact. Rev.* Vol. 3, No. 1, March 1968, pp. 55–83.

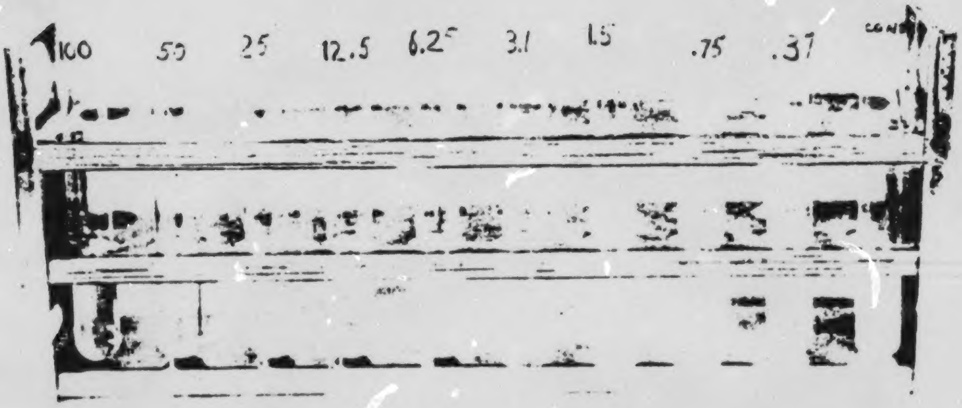


Figure 50. Dilution method of sensitivity testing.

15-9. A third mechanism was mentioned earlier for acquiring resistance, i.e., nongenetic variation or "physiological adaptation" to increased concentrations of a drug. There is support among some investigators for the concept that bacteria under certain circumstances can adjust their metabolic pathways or their complement of enzymes to compensate for the effect of harmful agents in the environment. This compensatory mechanism presumably operates without gene mutation and in the absence of phage transduction or conjugation.

15-10. At present our knowledge is limited pertaining to the specific reactions that take place when a resistant cell encounters a molecule of an antimicrobial agent. However, one or more of the following cellular states may play a part in conferring resistance, irrespective of whether drug tolerance in any given case has a genetic or an adaptive basis.[9]

a. Decreased penetration of the agent into the cell.

b. Increased destruction of the antimicrobial agent or its conversion to an inactive form (typical of the penicillinase enzymes).

c. Greater production of a metabolite or growth factor with which the drug (as a structural analog) competes.

d. Increased production of the enzyme that uses this metabolite (a higher concentration would be required to tie up all the enzyme molecules).

e. An alternate pathway that doesn't require the metabolite.

16. Determination of Bacterial Sensitivity

16-1. In the laboratory, you will perform sensitivity, or susceptibility, tests in order to predict the probable response of an infectious organism to drug therapy. The physician relies on the test to help him select an antibiotic for initial treatment; and, later, to detect the development of microbial resistance to that antibiotic during the course of the disease. As a further aid, as a laboratory technician, you can use sensitivity tests to measure the potency of a drug and to determine the concentration of an antimicrobial agent in selected tissues or body fluids.

16-2. In principle, the sensitivity test is simple. You cultivate an organism in either a liquid or solid medium containing an antimicrobial agent. After incubation, observe the culture for the presence or absence of growth (drug-resistance or drug-sensitivity), or as is usually the case, look for the degree of growth inhibition induced by several specific concentrations of a drug.

---

[9] Hedgecock, pp. 192-195.

16-3. **Laboratory Test Procedures.** There are two principal sensitivity test methods, although variations of these are frequently encountered. These are:

- Dilution method.
- Diffusion method.

16-4. *Dilution method.* In the dilution method, you prepare serially decreasing concentrations of an antimicrobial agent in tubes of a liquid medium. Add a uniform volume of a young, pure culture of the test organism to each tube. After overnight incubation, observe the tubes macroscopically for evidence of growth. A control tube containing the bacterial inoculum, but without the drug, is always included in the test system.

16-5. The tube having the lowest concentration of the drug that inhibits growth tells you the *minimal inhibitory concentration* (MIC). The MIC is usually expressed in micrograms of antimicrobial agent per milliliter. Figure 50 illustrates the tube dilution method and shows an MIC of 6.25 micrograms. As an alternate technique, you can incorporate dilutions of the inhibitory agent into agar slants or plates of a solid test medium. In this procedure, streak a loopful of inoculum on the surface, and then note the presence or absence of growth after incubation.

16-6. The dilution procedures yield quantitative results, and they are particularly useful in studying the action of drug combinations. The MIC of a single antimicrobial substance, or combination, can be compared to the therapeutic concentration known from clinical experience to be attainable in the body. Thus, the physician can judge in advance the probable efficiency of a given drug in controlling infection. There are certain disadvantages, however. The preparation of serial dilutions is too time consuming to have routine application in the clinical laboratory. In addition, you must first isolate the test organism in pure culture because the liquid culture system does not permit visual detection of contaminants. Resistant mutants that might develop in the broth tubes would be equally difficult to detect.

16-7. *Diffusion method.* The diffusion method takes advantage of the fact that antimicrobial drugs implanted at points on a solid agar medium will diffuse outward to give clearly recognizable zones of inhibition of bacteria growing on the medium. Perhaps the most widely used technique for routine work is *disc diffusion.* Several commercially manufactured products are available in the form of absorbent paper discs impregnated with antimicrobial drugs in known concentration. Discs are placed on seeded or surface-streaked plates of solid culture medium. Figure 51 shows the typical zones of inhibition that appear during incubation. This is caused due to moisture from the medium which dissolves the impregnated drug and permits it to diffuse outward from each disc. As we will note in the next section, there are precautions to take in spacing the discs properly and in inoculating the plate; but the convenience, flexibility, and time-saving features of the disc diffusion procedure make it well suited to the clinical laboratory.

16-8. **Factors in Sensitivity Testing.** Although none of the present-day techniques fully meets this goal in every respect, ideally, a good sensitivity test procedure should have all of the following characteristics:

a. Have a distinct end-point.
b. Be applicable to all therapeutic drugs.
c. Be suitable for use with all pathogens.
d. Give results in a short time.
e. Permit recognition of pathogens as opposed to contaminants.
f. Differentiate strains that are morphologically similar but unlike in drug sensitivity.

16-9. In actual practice, there are many variables in the technique of performing a sensitivity test. As a competent bacteriologist, you should strive to control these variables by setting up uniform procedures that will yield consistent results in the day-to-day operation of your laboratory. Among the factors which you must keep in mind are:

a. pH of the medium.
b. Composition of the medium.
c. Size and nature of inoculum.
d. Incubation period.
e. Growth requirements.
f. Stability of the drug.
g. Proper spacing of discs.

16-10. *pH of the medium.* Some antibiotics are more potent at one pH than another; for example, streptomycin shows its greatest inhibitory effect at an alkaline pH while penicillin is more effective at acid pH's. Nevertheless, the critical pH factor is the optimum range for growth of the test organism, usually 7.2 to 7.4. Moreover, this range encompasses the pH (around 7.35) encountered by the pathogen in the patient's circulatory system.

16-11. *Composition of the medium.* A heart infusion or tryptose blood agar base (with or without added blood) is ordinarily used in sensitivity testing. Not all culture media are suitable, however. Nutrients that will support growth of the fastidious pathogens must be provided. Conversely, substances that might interfere with the test must be excluded. Glucose in concentrations over 0.5 percent is not desirable, nor is the presence of serum albumin. This protein can absorb and inactivate certain penicillins. The blood cells in blood agar can also inhibit penicillin K. Since many tissue extracts contain p-aminobenzoic testing sensitivity to the sulfonamides (PABA would reverse any inhibition by these drugs). In addition to composition, the physical characteristics of solid media are important with respect to diffusion of the test antibiotics. The concentration of agar affects the rate of diffusion, as does the depth of plated media. Plates of uniform thickness (4 to 5 mm.) containing either 1.5 or 2.0 percent agar usually give the best results.

16-12. Size and nature of inoculum. Inoculum size is important with regard to two points: (1) the concentration of test microorganisms per volume of broth medium, or per given area of plate surface; and (2) the total coverage of a plated medium on which antimicrobial drugs will be implanted for the sensitivity test. A heavy inovulation is undesirable because the organisms, if present in large numbers, can "overgrow" a plate and mask the inhibitory effect of the test drugs. Similarly, a massive inoculum added to a drug-containing broth can quickly deplete the drug, and then multiply to yield a false result.

16-13. Complete coverage of a plate of culture medium with the incculum insures that all areas surrounding an implanted disc will contain

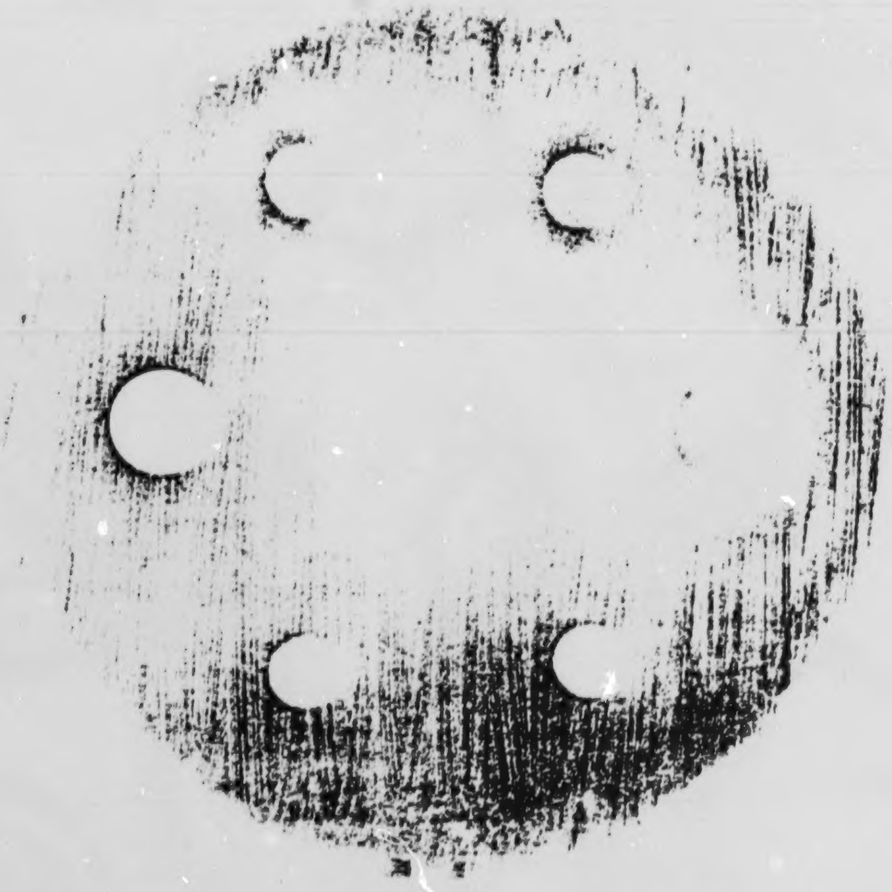


Figure 51 Correct technique of diffusion sensitivity testing.

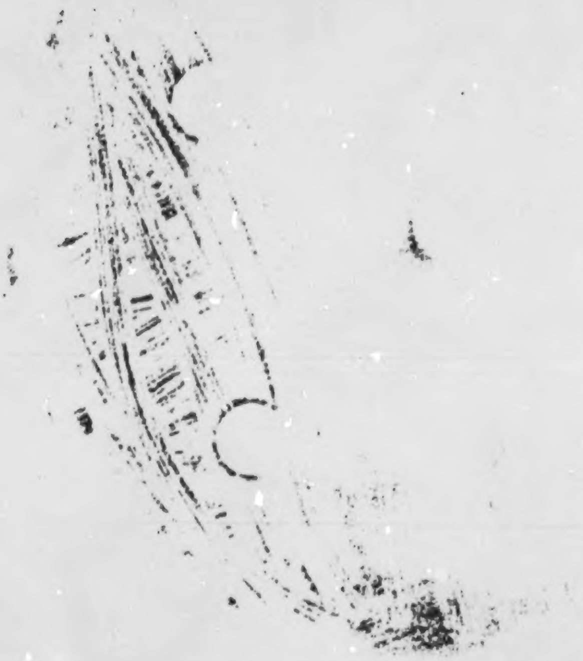


Figure 52. Disc diffusion—too heavily inoculated.

microorganisms. Otherwise, areas void of growth will mistakenly appear as zones of inhibition after the plate has been incubated. Generally, pure cultures of bacteria grown in broth for 2 to 5 hours, or until a visible turbidity is seen, provide the ideal inoculum. You can swab or flood this broth on plated media, or transfer it to tubed nutrient broth containing an antimicrobial agent, if a liquid system is preferred.

16-14. It quite often happens that treatment of an infection cannot be delayed until the pathogen is isolated in pure culture and identified. Under such circumstances, you have to set up sensitivity studies without waiting for the broth subculture to provide the ideal inoculum for diffusion tests. There are two shortcuts available to you, each having to do with the form of the inoculum.

16-15. The first of these involves the primary isolation medium to which the clinical specimen was inoculated. As soon as you can see macroscopically visible growth on a plate, select representative colonies for sensitivity testing. Biochemical, serological, or animal studies aimed at specific identification of the organisms can be carried out later. Using a loop needle, remove material from several colonies that seem to be identical in morphology. If the plate shows evidence of a mixed culture, sample separately the colonies of each distinct morphological type. When suspended and dispersed in a small volume of saline or broth, the material taken from multiple colonies is streaked or poured over the surface of the diffusion test medium. If you initially planted the clinical specimen in a fluid medium such as thioglycollate broth, you can use aliquots of this broth

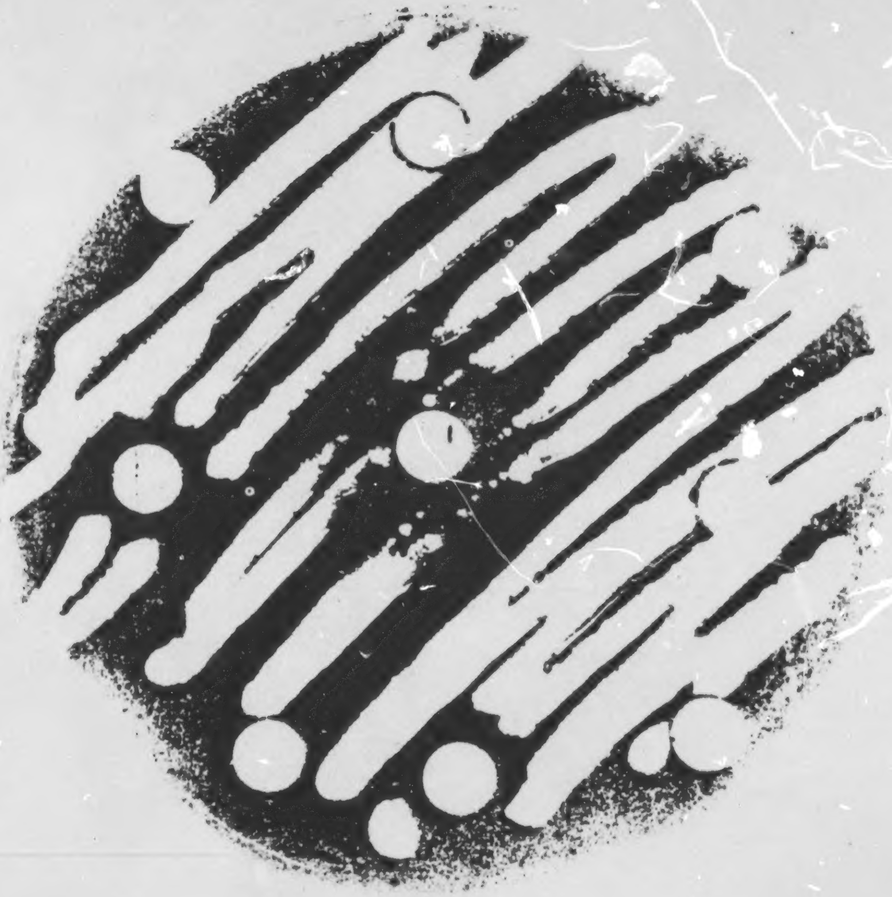


Figure 53. Disc diffusion—inoculum not uniformly applied.

for the sensitivity test inoculum as soon as you detect turbidity.

16-16. The second shortcut in inoculation techniques has been referred to as the primary disc diffusion procedure. After you have applied the clinical specimen to an isolation medium, use part of the specimen to inoculate the sensitivity test plate directly. You can spread urine, blood, and other body fluids over the surface with a glass rod or swab. Then, implant the discs containing antibacterial agents on the surface. The rapidity with which this inoculation technique gives results (generally overnight, but as short as 6 hours if the plate is examined microscopically) justifies its use in the hospital laboratory. Don't forget, though, to leave part of the inoculated surface free of the drug-impregnated diffusion discs so that colonies will be available for identification.

16-17. *Incubation period.* The optimum period of contact between test organism and the antimicrobial agent is 18 to 24 hours for most pathogens. As we noted above, minute colonies can sometimes be seen through a hand lens or microscope within a few hours after inoculation. Zones of inhibition are more clearly readable, however, if the longer period is employed. Beyond 24 hours the results may be obscured by overgrowth of microorganisms on the test plate or in tubes of liquid test medium. Many antimicrobial substances are bacteriostatic (rather than lethal) and prolonged incubation sometimes enables bacteria that were initially inhibited to counter the effects of the drug and to resume growth.

16-18. Many of the other considerations that we discussed earlier relative to cultivating bac-

106

teria in the laboratory also apply to sensitivity testing. If you find an obligate anaerobe in a diseased tissue, you must set up the sensitivity test system under anaerobic conditions, and at the proper temperature of incubation to support rapid growth of the organism. The same can be said for pathogens that require increased carbon dioxide pressure on first isolation.

16-19. *Growth requirements.* The tailoring of culture conditions to fit the organism's requirements for growth occasionally prove detrimental as far as the drug component of the test is concerned. The pH dependence of streptomycin and penicillin for maximum activity was mentioned previously.

16-20. *Stability of the drug.* The usual incubation temperature of 37° C. for pathogens inactivates certain of the antibiotics if the exposure is prolonged. Thus, drug instability, in addition to the other variables, accounts for the fact that our present-day sensitivity test techniques fall short of the ideal.

16-21. *Proper spacing of discs.* There is a last but extremely important factor to consider in conducting sensitivity tests by the disc diffusion technique—the proper spacing of the discs in relation to the bacterial inoculum. Let's look at the correct technique first, and then examine some examples of poor work. Referring to figure 51, you can see light but uniform coverage of the test plate with bacterial growth. The diffusion discs are placed far enough apart that zones of inhibition do not overlap. By contrast, the inoculation shown in figure 52 was so heavy that overgrowth of the antimicrobial agent occurred. Figure 53 demonstrates what can happen when the inocu-

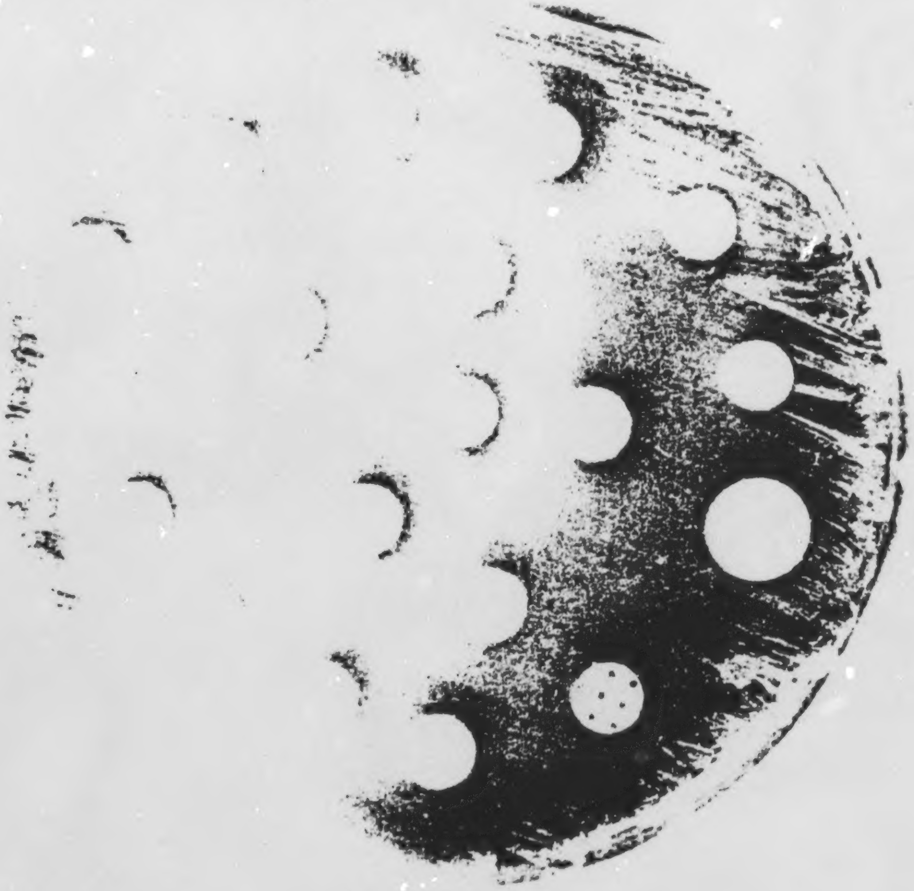


Figure 54. Disc diffusion—overcrowding of discs.

ium is not uniformly applied. It is difficult to determine with certainty whether clear areas around certain discs on the periphery of the plate are due to inhibition or to the absence of the inoculum. Overgrowth can also be suspected near other discs that give no sign of inhibition. At the other extreme, figure 54 reveals the result of crowding discs on the plate. The test becomes worthless when you try to find distinct zones of inhibition.

16-22. **Antibiotic Assays.** To assist in judging the level of antimicrobial drug in the patient's tissues, your laboratory may be called upon to assay blood, urine, or other body fluids for their drug content. The procedure is very similar to sensitivity testing. First, impregnate discs of absorbent paper with the patient's fluid to be assayed. Allow other discs to absorb a fluid of the same kind to which you have added several known concentrations of the drug in question. These discs serve as controls. Examine zones of inhibition on plates inoculated with an organism highly sensitive to that specific antibiotic. By comparing the size of the zone around the test disc to the zones around the discs of known drug content, you can arrive at a reasonable estimate of the drug level in the patient.

Bibliography

Books

BREED, R.S., E.G.D. Murray, and N.F. Smith. *Bergey's Manual of Determinative Bacteriology*, 7th Ed. Baltimore: Williams & Wilkins Co., 1957.

BRYAN, Auther H., C.A. Bryan, and C.G. Bryan. *College Outline Series—Bacteriology Principles and Practice* 6th Ed. New York: Barnes & Nobles, Inc., 1962.

CLIFTON, C.E. *Introduction to Bacterial Physiology*. New York: McGraw-Hill, 1957.

DORLAND, W.A.N. *The American Illustrated Medical Dictionary*, 21st Ed. Philadelphia: W.B. Saunders, 1947.

DUBOS, René J. and J. G. Hirsh. *Bacterial and Mycolic Infections of Man*, 4th Ed. Philadelphia: J.B. Lippincott Co., 1965.

FROBISHER, Martin. *Fundamentals of Microbiology*, 7th Ed. Philadelphia: Sanders Pub. Co., 1962.

GOODMAN, Louis S. and A. Gilman. (Editors). *Pharmacological Basis of Therapeutics*, 3rd Ed. New York: McMillan, 1965.

HEDGECOCK, Loyd W. *Antimicrobial Agents*, No. 3 in Medical Technology Series. Philadelphia: Lea V. Febiger, 1960.

LECHEVALIER, Hubert A. and M. Solotorovsky. *Three Centuries of Microbiology*. New York: McGraw-Hill, 1965.

OGINSKY, Evelyn L. and W.W. Umbrit. *An Introduction to Bacterial Physiology*, 2nd Ed. San Francisco: W.H. Freeman Co., 1959.

ROSEBURY, J. *Microorganisms Indigenous to Man*. New York: McGraw-Hill, 1962.

SALLE, A.J. *Fundamental Principles of Bacteriology*, 5th Ed. New York: McGraw-Hill, 1961.

SMITH, David T., N.F. Conant, and J.R. Overman. *Zinsser Microbiology*, 13th Ed. New York: Appleton-Century-Craft, 1964.

WILSON, Sir Graham S. and A.A. Miles. *Topley & Wilson's Principles of Bacteriology & Immunity*, Vol I & II, 5th Ed. Baltimore: The Williams & Wilkins Co., 1964.

Other Publications

ADM–40, *Techniques for Microbiological Analysis*, 1963 Ed., Millipore Filter Corp., Bedford Mass.

Difco Manual, 9th Ed. Difco Laboratories, Detroit, Mich., 1953.

ADM–10, *Millipore Application Data Manual*. Millipore Filter Corp., Bedford, Mass., 1964.

Spordex, American Sterilizer Co., 1965.

FEINGOLD, D.S. *New Engl J. Med*. 269:900, 1963.

MEYNELL, Elinor, G.G. Meynell, and Naomie Datta. "Phylogenetic Relationships of Drug-Resistance Factors and Other Transmissible Bacterial Plasmids." *Bact. Rev*., Vol 3, No 1, Mar. 1968, pp 55–83.

REGNAY, Peter P. "Chemistry of Antibiotics of Clinical Importance." *Amer. Jo of Med.*, Vol 18, No 5, May 1955, pp 686–716.

NOTE: None of the items listed in the bibliography above are available through ECI. If you cannot borrow them from local sources, such as your base library or local library, you may request one item at a time on a loan basis from the AU Library, Maxwell AFB, Alabama, ATTN: ECI Bibliographic Assistant. However, the AU Library generally lends only *books* and a limited number of AFM's. TO's, classified publications, and other types of publications are *not* available. (For complete procedures and restrictions on borrowing materials from the AU Library, see the latest edition of the *ECI Catalog*.)

Glossary

Animalcule—A minute, usually, microscopic organism.

Absorption—Taking up by capillary, osmotic, chemical, or solvent action. Examples: (1) absorption of moisture from the air, (2) absorption of gas by water, (3) absorption of nourishment in the small intestine, and (4) absorption by plant roots of nutrients from the soil.

Adsorption—A taking up by physical or chemical forces of the molecules of gases, of dissolved substances, or of liquids by the surfaces of solids or liquids with which they are in contact.

Autotrophic—Needing only carbon dioxide or carbonates as a source of carbon and a simple inorganic nitrogen compound for metabolic synthesis. (Obtains energy from the oxidation of inorganic compounds).

Aerobe—Organism that requires ready access to air for growth and reproduction.

Anaerobe—Organisms that can grow and reproduce in the complete or virtual absence of molecular oxygen.

Antigen—Any substance which, when introduced into the blood or tissues, incites the formation of antibody and which, when mixed with the antibody, reacts with it in some way that can be demonstrated.

Anthocyanin—Any of various soluble glucoside pigments producing blue to red coloring in plants.

Ascitic Fluid—Serous fluid in the abdomen.

Alanine—An amino acid formed by the hydrolysis of proteins.

Batericidal—Destroying bacteria.

Chemotherapeutic—Chemical agent used in the treatment of disease.

Coenzyme—The heat-stable, water soluble portion of an enzyme which is necessary for the production of fermentation or digestion by means of an enzyme.

Carotenoids—Yellow to red pigments found widely in plants and animals.

Cytoplasmic Membrane—A semipermiable membrane located directly beneath the cell wall, governing osmotic activity.

Epidemiology—A science that deals with the incidence, distribution, and control of disease in a population.

Enteric Organisms—Members of the family Enterobacteriaceae, many of which are parasitic and/or pathogenic in plants or in the intestinal tract of vertebrates.

Fulminating—Sudden, severe, coming on suddenly with an intense severity.

Halogen—Any element capable of forming a haloid salt. The halogens are chlorine, bromine, iodine, and fluorine.

Heterotrophic Bacteria—Bacteria that obtain energy from organic carbon sources.

Hydrolsate—A compound produced by a chemical reaction in which a compound reacts with water.

Isoniazid—A compound used in the treatment of tuberculosis.

Inspissator—An apparatus to make a fluid less thin by evaporation.

Lytic—Producing the dissolution of cells.

Lysochromes—Any of the naturally occurring pigments soluble in fats or in solvents for fats.

Mesophilic—Said of bacteria which develop best at the temperature of the body—37° C.

Microaerophilic—Preferring low concentrations of oxygen.

Melanin—A dark brown or black animal or plant pigment.

Metabolism—The chemical changes in living cells by which energy is provided for vital processes and activities and new material is assimilated.

Oxidation-Reduction—A chemical reaction in which one or more electrons are transferred from one atom or molecule to another.

Protophyta—A group of the vegetable kingdom, including the lowest and simplest plants, such as the bacteria.

Pleomorphic—Occurring in various distinct forms within the same species.

Psychrophilic—Fond of cold; bacteria which develop best between 15° and 20° C.

Penicillinase—An enzymelike substance produced by certain bacteria which has an inactivating effect on penicillin.

Pellicle—A thin skin or film on the surface of a liquid.

Proteolysis—The hydrolysis or decomposition of proteins.

Pathogen—A microorganism capable of causing disease.

Saprophyte—An organism which normally inhabits a certain area of the body without producing disease.

Spore—Unicellular resistant or reproductive body produced by plants and some invertebrates.

Taxonomy—Branch of biology that deals with the arrangement and classification of animals and plants.

Thermophilic—Fond of heat; bacteria which develop best at a temperature of 40° to 70° C.

AU GAFS,ALA.(753204)2200

10-3 112

90412 01 21

WORKBOOK

Laboratory Procedures in Clinical Bacteriology (Part I)

SELF-TEACHING AIDS
CRE
VRE
SRG

This workbook places the materials you need *where* you need them while you are studying. In it, you will find the Chapter Review Exercises and their answers, and the Volume Review Exercise. You can easily compare textual references with chapter exercise items without flipping pages back and forth in your text. You will not misplace any one of these essential study materials. You will have a single reference pamphlet in the proper sequence for learning.

These devices in your workbook are autoinstructional aids. They take the place of the teacher who would be directing your progress if you were in a classroom. The workbook puts these self-teachers into one booklet. If you will follow the study plan given in "Your Key to Career Development," which is in your course packet, you will be leading yourself by easily learned steps to mastery of your text.

If you have any questions which you cannot answer by referring to "Your Key to Career Development" or your course material, use ECI Form 17, Student Request for Assistance," identify yourself and your inquiry fully and send it to ECI.

Keep the rest of this workbook in your files. Do not return any other part of it to ECI.

EXTENSION COURSE INSTITUTE
Air University

TABLE OF CONTENTS

Study Reference Guide

Chapter Review Exercises

Answers to Chapter Review Exercises

Volume Review Exercise

ECI Form No. 17

STUDY REFERENCE GUIDE

1. *Use this Guide as a Study Aid.* It emphasizes all important study areas of this volume.

2. *Use the Guide as you complete the Volume Review Exercise and for Review after Feedback on the Results.* After each item number on your VRE is a three digit number in parenthesis. That number corresponds to the Guide Number in this Study Reference Guide which shows you where the answer to that VRE item can be found in the text. When answering the items in your VRE, refer to the areas in the text indicated by these Guide Numbers. The VRE results will be sent to you on a postcard which will list the *actual VRE items you missed.* Go to your VRE booklet and locate the Guide Number for each item missed. List these Guide Numbers. Then go back to your textbook and carefully review the areas covered by these Guide Numbers. Review the entire VRE again before you take the closed-book Course Examination.

3. *Use the Guide for Follow-up after you complete the Course Examination.* The CE results will be sent to you on a postcard, which will indicate "Satisfactory" or "Unsatisfactory" completion. The card will list *Guide Numbers* relating to the questions missed. Locate these numbers in the Guide and draw a line under the Guide Number, topic, and reference. Review these areas to insure your mastery of the course.

Guide Number	*Guide Numbers 100 through 125*
100	Introduction to History of Bacteriology; Major Contributions in the Discovery of Bacteria; Contributions of Important Bacteriologists, pages 1-3
101	Introduction to Aseptic Techniques and Sterilization Procedures; Procedures for Receiving and Collecting Specimens, pages 4-9
102	Handling Microbiology Specimens, pages 9-16
103	Sterilization and Disinfection: Sterilization Fundamentals, pages 16-19
104	Sterilization and Disinfection: Physical Agent Sterilization, pages 19-23
105	Sterilization and Disinfection: Chemical Sterilization; Chemical Salts, pages 23-27
106	Sterilization and Disinfection: Characteristics of a Good Disinfectant; Asepsis Testing; Practicing Aseptic Techniques, pages 27-32
107	Introduction to Bacterial Morphology and Physiology: Classification of Bacteria, pages 33-38
108	Cellular Morphology, pages 38-42
109	Cultivation of Bacteria: General; Growth Requirements; Physiology; Colony Morphology; Bacterial Pigments, pages 42-46
110	Cultivation of Bacteria: Anaerobic Cultures, pages 46-49
111	Culture Media and Environment: General; General Classes of Media, pages 49-51
112	Culture Media and Environment: Preparation of Media, pages 51-55
113	Culture Media and Environment: Media Additives; Biochemical Media; Biochemical Tests, pages 55-58
114	Introduction to Inoculation of Media for Isolation of Bacteria; Isolation and Cultivation of Bacteria, pages 59-62
115	Initial Isolation Procedures: General; Throat and Nasopharyngeal Swabs, pages 62-65
116	Initial Isolation Procedures: Sputum, Bronchial, and Gastric Washings for Acid-Fast Bacterial (AFB), pages 65-68

Guide Number

117 Initial Isolation Procedures: Body Fluids; Exudates, pages 68-71

118 Initial Isolation Procedures: Urine Specimens; Counting Techniques; Fecal Specimens, pages 71-75

119 Initial Isolation Procedures: Blood Specimens; Genitourinary Secretions; Tissue Specimens, pages 75-77

120 Stain Technology, pages 77-82

Guide Number

121 Introduction to Antimicrobial Agents, Drug Resistance, and Sensitivity Testing; Antimicrobial Agents, pages 83-85

122 Mode of Drug Action, pages 85-89

123 Mechanism of Antibiotic Resistance, pages 89-90

124 Determination of Bacterial Sensitivity, pages 90-96

125 Glossary, pages 99-100

CHAPTER REVIEW EXERCISES

The following exercises are study aids. Write your answers in pencil in the space provided after each exercise. Immediately after completing each set of exercises, check your responses against the answers for that set. Do not submit your answers to ECI for grading.

CHAPTER 1

Objective: To show a knowledge of some of the specific historical background of bacteriology and how it developed into the science it is today.

1. To be a good bacteriologist you must be a "good detective." In your own words, how does this apply to your job in bacteriology? (Intro.)

2. What practices did Hippocrates advocate for the "surgeon." (1-1)

3. In 1546, Girolamo Fracastoro proposed that contagious material can be spread in three ways. What are they? Do these ways still hold true today? (1-3)

4. What was the theory of *Spontaneous generation*? (2-1)

5. Spallazani, Schroeder Von Dusch, and Pasteur offered proof to discredit the theory of spontaneous generation. In your own words, briefly explain the bacteriological significance of this proof. (2-2-5, 8, 9)

6. Louis Pasteur was a major scientific contributor to the *Golden Age of Bacteriology*. What "title" has been bestowed upon Pasteur? (2-9)

7. List two reasons why a bacteriologist might not be able to use Koch's postulates in identifying a bacterium isolated from a disease source. (2-11)

CHAPTER 2

Objectives: To learn how to handle the various specimens submitted to the laboratory so that proper culturing techniques can be used, and to know how to maintain aseptic techniques and how to dispose of used materials.

1. Who is authorized to prepare the Standard Form (SF) 514 series requesting laboratory work? (3-2)

2. How can we prevent a specimen from getting separated from its request form? (3-4, 6)

3. When should blood specimens be collected for culturing? (3-8)

4. When submitting body fluids for culture, what information should be entered on the Standard Form 514? (3-11)

5. What is an exudate? List some sources from which they are usually collected? (3-12-14)

6. All food handlers must submit stool specimens for examination. Explain. (3-15)

7. How does sputum differ from spittle? (3-21)

8. Why should you, the laboratory technician, avoid touching the tongue, cheek, and teeth when collecting a throat culture? (3-23)

9. You have received a throat culture swab. The doctor has ordered a smear, culture, and sensitivity. You get all our materials ready, make the smear and plate the culture. What is wrong with this procedure? (3-24)

10. A physician has ordered three urine cultures on a patient over several days. What does he suspect? (3-25)

11. A three-glass urine is felt to separate a urine specimen into three anatomically separate specimens. What is the separation? (3-29)

12. If you are interested in identifying the bacteria which may be contained in a specimen, why concern yourself with material on the outer surface of the container? (4-1)

13. How does working on an absorbent surface such as a disinfectant soaked paper towel benefit you in the bacteriology laboratory? (4-4, 5)

14. What three things should you check when using the centrifuge? (4-6)

15. List at least five factors that you should take into consideration when selecting a specimen container? (4-9)

16. Body fluids may coagulate after being withdrawn from a patient. Describe three techniques you can use with a body fluid in order to culture it. (4-11)

17. The lapse of time between collection and media inoculation can be a detrimental factor in recovery of pathogenic bacteria. How can a holding media benefit recovery of bacteria? (4-16, 17, 32)

18. Discuss at least two reasons why you should initiate culture procedures as soon as possible after the collection of the material? (4-18)

19. Why must you be careful when freezing bacteria as a means of preserving them? (4-23)

20. In cases of suspected viral disease, two specimens are collected for diagnostic purposes. How are the tubes labeled and what is the significance of each specimen? (4-24)

21. Refrigeration at 4° C. is used as a means of temporary storing of bacteria-containing material. What is the main advantage of this means of preservation? (4-27)

22. When should you not use chemical preservatives to preserve bacterial cultures? (4-29)

23. Phenol is an excellent compound for surface decontamination. Why is it so good? (4-36)

24. What first aid should be given for a laceration in bacteriology? (4-41)

25. How does a laceration differ from a puncture wound in regards to bacterial contamination? (4-42)

26. Why is an accidental puncture with a needle more dangerous than a laceration? (4-42)

27. *What causes most accidental infection with contaminated fluids?* (4-43)

28. What causes most accidents while using pipettes? (4-45, 46)

29. Why should you not smoke or eat in the laboratory? (4-47)

30. Who do you protect when you make an effort to prevent contamination in the laboratory? (4-48)

31. You have sterilized all the equipment and media before using them in studying the bacteria isolated from clinical specimens. Why is this procedure important in your work? (5-1)

32. "The complete destruction or removal of all living forms of microorganisms" defines which term? (5-5)

33. What is the main difference between sterilization and disinfection? (5-5, 6)

34. Explain a bacteriostatic agent. (5-8)

35. Define the term "viability." (5-11)

36. How can we destroy viable organisms? (5-11)

37. What makes the genera *Staphylococcus* and *Bacillus* differ from each other enough to warrant the use of a different means of sterilization for each one? (5-13)

38. In which part of the growth cycle would spore-forming organisms be harder to kill? (5-15)

39. How does the presence of organic matter affect the effectiveness of disinfectants? (5-16, 18)

40. Disinfectants do not act immediately. The speed with which they react depends mainly on what four factors? (5-17)

41. Increased moisture and an elevated temperature affect bacteria in two stages. What are they? (5-22)

42. What factors affect the effectiveness of boiling as a means of sterilization? (5-23, 24)

43. What is the principle of the Arnold sterilizer method of using free-flowing steam? (5-28, 29)

44. What time, temperature, and pressure are routinely used to sterilize bacteriology laboratory materials in an autoclave? (5-32)

45. Dry heat can be used to sterilize glassware and nonliquid materials. What important factors must be considered? (5-33-35)

46. List four types of filters that can be used as a mechanical means of sterilization. (5-38)

47. How does a filter achieve sterilization of liquids? (5-40)

48. Chemical sterilization is achieved by attacking what three sites of a bacterium? (5-42)

49. How do surfactant chemical agents affect the cell? (5-44)

50. Match the substances on the left with the ways they affect bacteria listed on the right. (5-44-62)

1. Sulfonamides.
2. Halogens.
3. Heavy metals.
4. Synthetic detergents.
5. Organic solvents.

a. Kills vegetative stages.
b. Coagulation of cell protein.
c. Interfere in enzyme processes.
d. Precipitation of various proteins and agglutination of bacteria.
e. Nonspecific chemical combinations.

51. Which halogen is the most dangerous to use? (5-51)

52. What kind of bacteria is more susceptible to detergents? (5-55)

53. Name the three types of detergents. (5-55)

54. Halogens are one of the classic means of disinfection. Recently synthetic detergents have gained wider acceptance and more use. How do they act on cells? (5-55)

55. Match the type of detergent on the left with the statement on the right. (5-56-58)

(1) Anionic.	*a.* Active at alkaline pH.
(2) Cationic.	*b.* pH independent.
(3) Nonionic.	*c.* Active at acid pH.

56. What can be added to ethyl alcohol to increase its effectiveness against spores? (5-61)

57. A practical means of sterilizing plastic articles is by the use of which type agent? (5-67)

58. Phenol is commonly used in what percentage strength? (5-69)

59. The addition of ethyl alcohol to phenol solution will *increase or decrease* the effectiveness of the phenol. (5-69)

60. To offer standardization in measuring the effectiveness of disinfectants, which agent is used as the standard? (5-71)

61. If we wanted to test the effectiveness of a disinfectant, what three conditions must we observe in culturing the organisms to be used in the test? (5-72)

62. When can we consider a phenol coefficient valid? (5-73)

63. What is the best disinfectant for smooth laboratory tabletops? (5-76)

64. How do we prevent hospital-borne infections? (5-77)

65. Who coordinates activities within USAF hospitals for the prevention and control of hospital-borne infections? (5-78)

66. There are three main techniques for testing the sterility activity of the autoclave. What are they? (5-80)

67. Which method of sterility testing is the most reliable indicator of proper operation of sterilization equipment? (5-80, 83)

68. In using the spore strip or ampule as a means of testing autoclave effectiveness, would growth of the spores after autoclaving indicate that the autoclave is effective or noneffective? (5-83-86)

69. When used as a sterility check ampules of *Bacillus stearothermophilus* must be incubated at what temperature? (5-85)

70. How should you interpret cultures of "clean" hospital rooms? (5-86)

71. If you are monthly sampling a floor for bacteria, why should you standardize your sampling by taking the specimen from one particular area, plant it on one kind of media and grow it in the same environment each time? (5-87, 88)

72. If you were to sum up the list of responsibilities that you have as a bacteriologist, what statement might you make? (5-93)

CHAPTER 3

Objective: To learn how taxonomy, morphology, and physiology of bacteria are useful parameters in identifying bacteria. To demonstrate a knowledge of the basic techniques of good culturing and biochemical testing as aids in speedy identification of organisms from clinical specimens.

1. What is a taxis? (6-2)

2. The most commonly used method for classifying bacteria is the *Code of Nomenclature of the Bacteria and Viruses.* What properties does this code use to classify bacteria? (6-4)

3. Arrange the following taxonomic categories in descending sequence. (6-5, 6)

a. ______________________ phylum

b. ______________________ kingdom

c. ______________________ genus

d. ______________________ family

e. ______________________ order

f. ______________________ tribe

g. ______________________ class

4. Identify the genus variety and species names of *Bacillus subtilis niger*? (6-7-12)

5. In your work you have cultured an organism, but you are not sure to what genus it belongs. What approach should you take in identification? (6-9)

6. What two terms are commonly used to designate a subspecies? (6-12)

7. A group of related genera is a ______________________. (6-13)

8. What features do we generally include under the term "morphological characteristics?" (6-18)

9. What are the three principal shapes of bacteria? (Table 4)

10. Bacteria which obtain their energy from inorganic material and their carbon from carbon dioxide are called ______________________bacteria. Those organisms which require organic carbon are called ______________________bacteria. Disease-producing bacteria are usually (heterotrophs/autotrophs). (6-20-22)

11. The unit used for measuring bacteria is the ______________________. (7-2)

12. The structure of a bacterial cell which contains diffused chromatin material responsible for the reproduction of the cell is the ____________________. (Fig. 15; Table 5)

13. A bacillus with flagella completely surrounding the cell is termed ________________. (Fig. 17)

14. The post-fission whipping of bacilli results in the arrangement referred to as ________________. (7-4, Table 6, Fig. 17.)

15. The genera representing cocci in fours and in packets of eight are ________________ and ________________ respectively. (7-6; Table 6)

16. As a result of binary fission, bacilli are seen in various groupings. Define the following terms which describe the appearance of the bacilli microscopically. (Table 6)

a. Bacillus.
b. Diplobacillus.
c. Streptobacillus.
d. Palisades.

17. Explain briefly the two types of variation. (7-9)

18. Mutation from penicillin-sensitive to penicillin-resistant is an example of what type mutation? (7-10)

19. Cultures can contain a mixture of typical and mutant bacteria. If you grew a culture on a medium which contained an antibiotic to which the typical bacteria were susceptible, what would you be apt to see after incubation? (7-10)

20. Change from smooth "S" colonies to rough "R" colonies is an example of ______________________, the second form of variation. (7-11)

21. What is pleomorphism? (7-13)

22. List the five factors that you must consider in cultivating bacteria. (8-3)

23. The majority of human pathogenic organisms grow best at a temperature of 37° C. and are called ______________________________. (8-7)

24. Define a facultative anaerobe. (8-12)

25. Define a microaerophil. (8-14)

26. In examining colonies of bacteria, what physical macroscopic characteristics should you observe? (8-15)

27. An H-type colony is the result of active flagellar motion. You would expect this type of colony to be (spread out/pinpoint). (8-17)

28. The terms "rough," "smooth," "heaped," and "mucoid" refer to which characteristics of the colony? (8-21)

29. A heavily encapsulated organism might be expected to form a ______________________ colony. (8-21)

30. Describe an S-type colony and an R-type colony. (8-22, 23)

31. Does the type of medium used to cultivate a bacterium influence the color produced by a colony? (8-24)

32. What are the two major groups of pigments that you may encounter when studying bacterial pigments? (8-25)

33. Name three methods by which you might obtain complete anaerobiosis. (8-28, 30-35)

34. A candle jar increases the carbon dioxide content of the enclosed air by approximately what percent? (8-29)

35. List at least three growth-promoting substances which should be incorporated into a culture medium. (9-3)

36. Under what specific test conditions would we omit carbohydrates from blood agar? (9-4)

37. What amount of agar should be added to a liquid base medium for optimum gelling? (9-5)

38. What are the four principal types of media? (9-9)

39. Differentiate between basal media, enriched media, and isolation media. (9-9-12)

40. Differential and selective media are subclassifications of an isolation medium. How do they differ from each other? (9-13, 14)

41. Why should you use fresh distilled water for reconstituting dry medium? (9-17)

42. You must watch for numerous points when preparing media. Match the correct response with the media or equipment. (9-17-24)

a. Gelatin.

b. Two liter flask.

c. 121° C., 20 to 30 min.

d. Filtration.

e. Enrichment materials.

f. pH.

1. Use to prepare 1 liter of finished medium.
2. Does not destroy heat-sensitive ingredients.
3. Dissolve by heating to 50° C. in hot water bath.
4. Optimum sterilization time and temperature.
5. Should be checked when you are using a formula and stock chemicals.
6. Used to prepare semisolid stab cultures.
7. Are added after cooling sterilized medium to 45° to 55° C.
8. Are used in small aliquots.

43. Why are bubbles not desirable on agar surfaces? (9-27-29)

44. Occasionally, we prepare a batch of blood agar which is lumpy. How did this happen and what is wrong with using lumpy medium? (9-31)

45. Should an experienced technician assume that his newly poured plates are sterile? Why? (9-37)

46. Since moisture is necessary for bacterial growth, can we use a medium which has water droplets on its surface to isolate bacteria? Why? (9-37)

47. A great many substances can be added to media for special purposes. For instance, sodium thioglycollate permits the growth of anaerobic bacteria because of its reducing action. Briefly state the reason for using the following additives. (9-38-47)

   a. Penicillinase.
   b. Crystal violet.
   c. Sodium desoxycholate.
   d. Potassium tellurite.
   e. Antibiotics.

48. How can you use carbohydrate fermentation studies as an aid in identifying genera and species of bacteria? (9-49, 50)

49. What three sugars are found in triple sugar iron agar? (9-51)

50. Para-dimethyl-amino-benzaldehyde (Kovac's reagent) is useful for determining the production of what chemical in bacterial cultures? (9-55)

51. What is the major difference between the principle of the MR and VP test procedures? (9-57, 58)

52. Proteolytic enzyme production can be detected by what test procedure? (9-59)

53. In the litmus milk fermentation test, what sugar is fermented to give a positive test? (9-62)

CHAPTER 4

Objective: To learn how to use a methodical scheme, or approach, in culturing various clinical specimens in order to identify microorganisms as carefully and as rapidly as possible. To understand how to properly prepare and stain smears of the specimens and cultures to gain the information needed for correct identification of microorganisms.

1. What major problem in diagnostic bacteriology do we face today that the bacteriologist Koch faced in the 1880s? (Intro.)

2. How does the surface streaking technique used on plate cultures help in the isolation of a bacterium? (10-3)

3. If we do not carefully "pick" a colony during subculture procedures, what might happen? (10-4, 5)

137

4. In what instance would we most likely use a pour plate in isolating a bacterium for identification? (10-8)

5. What do you do about the water present at the base of an agar slant? (10-11)

6. List the three types of tubed media and briefly state the use of each? (10-9-12)

7. What is the purpose of diluting certain clinical speciments before inoculation? (10-13)

8. What mechanism allows bacteria to grow to colony size on the membrane used in filter cultures? (10-14)

9. What is the reason for using a blood agar streak or pour plate for the initial isolation of organisms from a throat culture? (11-3)

10. How can we test for "O" hemolysin produced anaerobically by streptococci without doing a pour plate culture? (11-4)

152

11. Why would one wish to use enteric media for throat culture? (11-9)

12. In what sequence of processing steps should a sputum specimen submitted for both routine and AFB cultures be handled? (11-13)

13. Tubercle bacilli from an active case are highly infectious. What can we do in the bacteriology laboratory to prevent becoming infected? (11-15)

14. What is the purpose of the concentration technique in processing sputum for TB isolation? (11-16)

15. Give the advantages of using trisodium phosphate as a digestant of sputum. (11-19)

16. Middlebrook 7H10 agar is rapidly becoming a favorite medium to grow tubercle bacilli. What is a major disadvantage to the use of this medium? (11-24)

17. Clotting is a problem in processing body fluids for culture. What can we add to the fluid specimen to prevent clotting? (11-27)

139

18. India ink can be used to outline the capsule of____________________________ ____________________.
(11-29)

19. Exudates can orginate in many parts of the body. What must you know about this type of specimen before you can culture it? (11-31-33)

20. Significant bacteriuria is indicated by what criteria when culturing urine? (11-36)

21. After streaking an agar surface with 0.1 ml. of a 1:1000 dilution, you count 64 colonies. What is the count of bacteria per milliliter? (11-38)

22. To process a 24-hour pooled urine specimen for tubercle bacilli, one should add what reagent to the urine? What steps must follow? (11-41)

23. Why inoculate selenite-F broth or tetrathionate broth with feces when performing fecal cultures? (11-45)

24. How can knowledge of the patient's clinical diagnosis benefit the bacteriology technician when culturing a stool specimen? (11-46-49)

154

25. What is SF medium used for? (11-51)

26. Ideally, you should draw a blood culture before the start of antibiotic therapy. However, what can you do if this is not possible? (11-57)

27. A smear of a urethral secretion which shows many leukocytes and Gram-negative intracellular diplococci suggests what infection? (11-59; Fig. 40)

28. How should tissue specimens be cultured? (11-61-63)

29. List the three important points to remember when you prepare smears. (12-4)

30. What is the proper way to prepare a smear from a swab which appears to be dry? (12-7)

31. Slides should "air dry" before staining. How then should you "fix" a slide and why? (12-10)

32. A chemical group that gives the dye molecule its characteristic color is called a ________________. (12-12)

33. A substance which fixes (intensifies) a dye to a cell, thereby minimizing the dye's removal from the cell, is referred to as a ____________________________. (12-13)

34. What is the difference between a direct and an indirect stain? (12-14)

35. If we want to demonstrate the presence of a certain feature of a bacterium (e.g., spore or capsule) one should use a ____________________________stain. (12-14)

36. Bipolar staining shows what feature of an organism? (12-15)

37. Three major factors influence the final color pattern on a properly stained smear. What are they? (12-16)

38. Although the mechanism of the Gram stain reaction is not fully understood, it is believed that the property of a cell's being Gram-positive lies in what part of the cell? (12-19)

39. (Gram-negative/Gram-positive) cocci may appear as a result of autolysis, aging, and improper incubation temperature. (12-21)

40. What would you suspect if on microscopic examination of a stained smear you noticed heavy debris and misleading artifacts along with the bacterial cells? (12-22)

41. What is the action of phenol in the carbol-fuchsin solution as employed in an acid-fast stain? (12-24)

42. What is the major difference between the Ziehl-Neelsen and the Modified Kinyoun acid-fast staining techniques? (12-25)

43. What feature of the carbon particles in India ink must we watch for? (12-26)

44. How can we protect India ink against bacterial contamination? (12-26)

45. Why is serum or plasma used in the "hiss" method of staining capsules? (12-27)

46. How is spore staining achieved when using the Wirtz-Conklin staining method? (12-28)

47. What is the purpose of the hanging drop method for examining wet preparations? (12-29)

48. What is the difference between flagellar motion and Brownian movement? (12-29)

CHAPTER 5

Objective: To gain an understanding of the way in which antibiotics and other therapeutic agents affect bacteria; and to learn the principles of measuring microbial sensitivity or resistance to drugs in the laboratory.

1. What is a chemical substance produced by living forms, and used as an antimicrobial agent, called? (13-1)

2. What is meant by selective toxicity of drugs? (13-3)

3. Antimicrobial agents affect bacterial cells by disrupting five functions of the cell. What are these five functions? (13-7)

144

4. Is an antimicrobial substance which inhibits the growth of an organism bacteriostatic or bactericidal? (13-9)

5. The cell wall building block of bacteria is composed primarily of what chemical substances? (14-2)

6. What is the primary function of "peptide linkage" in the bacterial cell? (14-3)

7. Is the osmotic pressure inside the bacterial cytoplasmic membrane higher or lower than the outside pressure? (14-4)

8. In simple terms, explain why the cell wall mucopeptides function imperfectly when a cell is subjected to an environment containing cycloserine. (14-6)

9. Most penicillins, although similar chemically, differ in what aspect of their molecular makeup? (14-7)

10. What is the role of the cytoplasmic membrane in the bacterial cell? (14-9)

11. What explanations have been given for the action of an antimicrobial agent on the cytoplasmic membrane? (14-10)

12. Why are some antimicrobial agents effective against pathogenic fungi and not bacteria? (14-12)

13. What is the function of soluble ribonucleic acid? (14-18)

14. What is the purpose of the messenger ribonucleic acid in the bacterial cell? (14-19)

15. Which refers to the buildup of cell constituents from simpler substances, anabolism or catabolism? (14-25)

16. Which of the below is/are competitive analogs? (14-27)

a. PABA.
b. PAS.
c. Sulfonamide.

17. What is it that makes many *Staphylococcus* organisms resistant to penicillin? (15-1)

18. Can drug tolerance be transferred among species? (15-2)

19. What two major genetic mechanisms within a bacterial cell allow an organism to develop a resistance to a particular antimicrobial agent? (15-3)

20. What is the difference between transformation and transduction as these processes take place in a bacterial cell? (15-4, 5)

21. How does conjugation differ from transformation and transduction? (15-4, 6)

22. Sensitivity or susceptibility test procedures are based on what two principles of methodology? (16-3)

23. What methods used for antibiotic sensitivity tests yield quantitative results? (16-6)

24. Ideally, what are the six properties that a good sensitivity test method should have? (16-8)

25. What are the variable factors in sensitivity testing that the technician must try to control? (16-9)

26. Glucose in concentrations greater than what percent may interfere with some antibiotic sensitivity testing? (16-11)

27. Why is a heavy inoculum undesirable when streaking for sensitivity testing? (16-12)

28. What two recognized shortcuts are available when a faster than routine sensitivity result is required? (16-15, 16)

29. Why should sensitivity studies be read for results within 18 to 24 hours after inoculation? (16-17)

30. What is the difference between antibiotic sensitivity testing and antibiotic assays? (16-21)

ANSWERS FOR CHAPTER REVIEW EXERCISES

CHAPTER 1

1. As a bacteriology technician and "detective," I must be able to gather facts about the morphology, physiology, and in-vitro growth of bacteria. I need to interpret what I see and sort through all the information gathered in the laboratory in order to come to a definite conclusion as to the name of an organism. (Intro.)
2. He stressed the use of boiled water for irrigating wounds and the importance of clean hands and fingernails of the "surgeon." (1-1)
3. Contacts, fomites, and from a distance (air currents). These methods of spreading contagious material are as true today as they were in the days of Fracastoro. (1-3)
4. Spontaneous generation was a theory that living organisms could originate from nonliving materials. (2-1)
5. These men offered proof that bacteria could be excluded from many materials if air currents did not transport airborne bacteria to the material. The use of sealed flasks, cotton or gause flask plugs, and the swan-necked flasks helped to prevent the spread of contagious material into the sterile material. The disproving of this theory has resulted in the wide use of the sealed containers in the canning industry. (2-2-5,8,9)
6. The Father of Bacteriology. (2-9)
7. *a.* Many organisms do not produce a human disease in a susceptible animal.
   b. An organism at times needs to be associated with another organism in order to produce a disease. (2-11)

CHAPTER 2

1. Patient's physician, ward nurse, senior medical service technician, or similar personnel assigned to the clinic are authorized to prepare SF 514 series. (3-2)
2. The container should be properly labeled to show the name of the patient, register number, ward location, and any pertinent identifying data. Double check to see that the container label and request slip show the same information and that the two are from the same patient. We may have two patients with the same name. Also look at the specimen source, sex, and procedures requested—are they the same? In short, be positive that all information is identical and plausible. Paragraph 3-6 gives some rules for handling cultures. (3-4-6)
3. When symptoms indicate circulatory involvement, such as chills, fever, or convulsions. (3-8)
4. The specimen source, provisionsl diagnosis, and any antibiotic therapy. (3-11)
5. An exudate is a material that has passed through the walls of vessels into adjacent tissues or areas of inflammation. It can be collected from boils, ear infections, eye infections, and cases of urethritis. (3-12-14)
6. Food handlers in public restaurants serve many people. It is possible for them to harbor various communicable intestinal organisms. They, themselves, do not have the disease, but can transmit the organisms to food and thereby infect a great number of people with para-typhoid or typhoid fever. (3-15)
7. Sputum is a secretion brought up from the lungs and bronchial tree. Spittle is generally saliva. (3-21)

8. You are trying to determine the organism causing an infection. If we touch these areas, you might pick up organisms that are not causing the throat problem. The bacteria in the mouth are probably normal and will only cause confusion in the isolation of the pathogen in the throat. (3-23)

9. If the smear is made first, it is possible to contaminate the specimen by transferring material from the slide to the specimen. Slides are not generally sterile. Certainly you don't want to add organisms to the ones on the swab. This invalidates any work you have done. (3-24)

10. A physician might suspect that an organism reported on a urine culture is a contaminant. He has ordered two others to verify the presence of the organism in the urinary system. He may subsequently order colony counts to check the number of organisms present. To further isolate the origin of the organisms, he might order a series of catheterized specimens to check where the organism might be in the urinary system. (3-25)

11. Of course, the urine must be a clean void. Insure that the patient understands how to cleanse the genitals before collecting the specimen. The first part of the specimen can indicate a urethral infection; the second part—an infection of the bladder; and the third part from the anterior part of the bladder, gives a specimen of urine which may indicate an infection in the ureters and the kidney. (3-29)

12. The contaminated exterior surface constitutes a source of infection to the handlers. Highly infectious material should be carefully handled. If it comes from a treatment area, it should be wrapped in some manner to prevent contaminating a whole area and certainly the handlers. After you have cultured it, autoclave the whole package. This will insure that there will be no contamination. (4-1)

13. "An ounce of prevention is worth a pound of cure!" It helps to minimize contamination caused by droplets, and aerosol, and kills bacteria which fall on the toweling. This prevents contamination again—of you, your work area, and your coworkers. And of course, an excellent practice is to wipe down the work area with a disinfectant when you have finished your work. (4-4, 5)

14. *a.* That the tubes are fitted with a tight lid to prevent contamination of the specimen or atmosphere.
    b. That the tubes are not chipped or cracked.
    c. That the centrifuge is balanced.
    (4-6)

15. *a.* Type of specimen.
    b. Source of specimen.
    c. Analysis desired.
    d. Time lapse between collection and inoculation.
    e. Final disposition of container.
    (4-9)

16. The three approaches which might be taken are:
    a. Allow the material to clot and then culture the entire clot.
    b. Use a sterile anticoagulant to prevent the material from clotting.
    c. Perform initial inoculation at the place of collection to avoid clotting.
    (4-11)

17. A holding media has many advantages. A few of these are as follows:
    a. To prevent swabs from drying out.
    b. Convenience of personnel for delivery.
    c. Preserve the viability of the bacteria.
    d. Maintain ratio of bacteria as when collected.
    e. To ship specimen.
    (4-16, 17, 32)

18. *a.* Specimens may dry out, allowing many of the bacteria to die.
    b. Certain fluids will change pH due to action of growing bacteria. This change in pH is detrimental to certain organisms.
    c. The original ratio of bacteria will be altered. Fast growing nonpathogens will overgrow slow growing pathogens. This may give a false indication as to the predominating organism.
    (4-18)

19. As bacteria are frozen, crystals tend to form within the bacterial cell. These crystals, in turn, may puncture the cell and cause destruction. Bacteria must be frozen under controlled conditions. (4-23)

20. The tubes are labeled "acute" and "convalescent." "Acute" refers to the specimen collected while the disease is in process. "Convalescent" refers to the specimen collected after the disease has subsided and antibodies have had a chance to build up within the body. The results of both specimens are compared and either one separately has no meaning in relation to the course of a disease. (4-24)

21. The metabolic process of the bacteria is slowed to a point where multiplication is almost impossible. This tends to retain the original number of bacteria. (4-27)

22. Chemical preservatives should never be used to preserve bacterial specimens. (4-29)

23. Phenol is absorbed as a thin fairly durable film on surfaces to which it is applied. A residual of phenol serves to destroy bacteria for several hours after application. (4-36)

24. Rinse the area well with running water, wrap with sterile gauze or suitable material, then seek medical aid. (4-41)

25. A puncture wound is usually deeper than a laceration and has less bleeding. As a result, bacteria are forced deeper into the tissue where they have a better chance for survival. (4-42)

26. A needle wound is more dangerous because there is no bleeding and microorganisms may be forced deep down into tissues. Bleeding washes microorganisms out of wounds and there is little infection if the bleeding is stopped aseptically. (4-42)

27. Carelessness and negligence. (4-43)

28. Too much suction and dirty pipettes. (4-45, 46)

29. It is not safe. Most people have a habit of putting things down for a while. If you lay a cigarette on a laboratory tabletop you run a great risk of getting an infection. Remember, bacterial contamination is not visible to the naked eye. (4-47)

30. Yourself, of course. Your family, in that you might take an infection home with you. Your co-workers who work with you. And, finally other members of the hospital staff and patients who might have to come into your work area. (4-48)

31. Bacteriological identification requires, that pure cultures of microorganisms be studied. Unsterile material will contain contaminating organisms. (5-1)

32. Sterilization. (5-5)

33. Sterilization means the complete destruction of the vegetative and spore stages of organisms. Disinfection may not kill the spores, thus not affecting complete sterilization. (5-5, 6)

34. An agent which does not cause immediate death of an organism, but rather acts to prevent multiplication of the organisms. (5-8)

35. Viability means the ability to live. A viable organism is capable of fulfilling all of life's processes. (5-11)

36. By altering either the physical or the chemical surroundings of the organism or both, thereby, affecting its life processes. (5-11)

37. Biochemical behavior. More specifically, the genus, Bacillus is a spore producer as the environment changes. If all the factors are not favorable to growth, cells will develop spores until more favorable conditions are available. The genus *Staphylococcus* does not develop spores, and therefore is easily killed by disinfectants. Bacillus needs to be sterilized by heat and pressure (autoclaving). (5-13)

38. At the end of the stationary and all of the senescent phase. (5-15)

39. Organic matter in solution with bacteria will readily combine with the disinfectant. As a result, the bacteria may not be harmed by the decreased effectiveness of a disinfectant. You may have to use a stronger disinfectant for a longer period of time to actually kill the bacteria. (5-16, 18)

40. *a.* Concentration of organisms.
    b. Concentration of disinfectant.
    c. Temperature of disinfectant.
    d. Presence of cells in the culture having varying susceptibilities.
    (5-17)

41. *a.* Water reacts with the protein. (Denaturation)
    b. The altered or denatured protein coagulates with heat.
    (5-22)

42. Time and temperature, both of which are related to altitude (pressure). (5-23, 24)

43. The Arnold Sterilizer uses the principle of Tyndall, whereby alternate heating and incubation destroy vegetative forms and allow spores to germinate to vegetative stages and then to be destroyed by successive heating. (5-28, 29)

44. 15 minutes, 121° C., and 15 pounds. (5-32)

45. *a.* Don't overload the oven.
    b. Allow plenty of room for the hot air to circulate between the articles and the walls of the oven.
    c. Follow the recommended times and temperatures for the oven. It might also be suggested that you time the heating cycle after the oven has reached the temperature recommended.

46. *a.* Berkefield–"infusorial earth."
    b. Chamberland–"unglazed porcelain."
    c. Pyrex brand–"fritted glass."
    d. Seitz filters–"compressed asbestos pads."
    (5-38)

47. A filter achieves sterilization by absorbing microorganisms on the surfaces of the filter. Therefore, you must be sure the filter is sterile prior to its use. (5-40)

48. *a.* Surface layer.
    b. Nuclear material.
    c. Enzyme systems.
    (5-42)

49. Surfactants coat the cell wall of the bacteria and prevent it from absorbing or utilizing nutritional materials. (5-44)

50. 1*c*, 2*e*, 3*b*, 4*d*, 5*a*. (5-44-62)

51. Bromine. (5-51)

52. Gram-positive bacteria. (5-55)

53. Anionic, cationic, and nonionic. (5-55)

54. Detergents are strong surface active agents. They are nonirritating and in fairly weak solution are still effective against vegetative forms of bacteria. They act to precipitate various proteins and agglutinate bacteria. The detergents are relatively more bacteriostatic than bactericidal. They are essentially three types–anionic, cationic, and nonionic. (5-55)

55. 1*c*, 2*a*, 3*b*. (5-56-58)

56. Acid such as sulfuric, or a base such as sodium hydroxide can be added to a 70 percent ethanol solution increases its effectiveness. (5-61)

57. Ethylene oxide. (5-67)

58. Five percent. (5-69)

59. Decrease. (5-69)

60. Phenol (carbolic acid). (5-71)

61. We must use a single kind of media; a single environment for growing the bacteria and the temperature must be the same. (5-72)

62. Only when all conditions and procedures in the test are standardized and controlled. (5-73)

63. An emulsified disinfectant or a liquid disinfectant containing a wetting agent. (5-76)

64. To prevent hospital-borne infection, we must use aseptic techniques as routinely as possible. Also, the hospital must have a program of aseptic testing. (5-77)

65. The Hospital Infection Committee. (5-78)

66. *a.* Use of heat-sensitive indicators.
    b. Testing the equipment, itself, by culturing.
    c. Use of spore strips or ampules. (5-80)

67. The spore strip method. (5-80, 83)

68. Noneffective. (5-83-86)

69. 55° C. (5-85)

70. All organisms in the culture must be identified. If they are saprophytes, the room is clean and can be used for the next patient. If we isolate pathogens, which may be from the previous patient, the room must be cleaned and disinfected again and the room recultured to make sure that all the dangerous organisms have been killed. (5-86)

71. Sampling technique must be standardized because no hospital is sterile, and a certain number of potential pathogens such as *Staphylococcus aureus* will be found on floors at all times. Your testing will indicate the number of organisms present when the floor is clean, and an increase would indicate the need for additional cleaning. If your tests indicate a change in organisms present on the floor and these were similar to the pathogens of a previous patient, it would indicate a need for recleaning. (5-87, 88)

72. Be careful and thorough in all your technical work! It is important to examine your procedures and techniques to be sure that you are doing all you can to isolate organisms. You must be careful to insure that you are helping to solve a problem and not contributing to it. (5-93)

CHAPTER 3

1. A taxis is an "arrangement." In our frame of reference, it would be an orderly arrangement or sorting of microorganisms within an organized classification system. (6-2)

2. All known properties of an organism. The properties include, shape, grouping, motility, Gram-stain reaction, and growth requirements. These fall into roughly three categories–morphology, physiology of life processes, and a miscellaneous group of characteristics. (6-4)

3. *a.* Kingdom.
   b. Phylum.
   c. Class.
   d. Order.
   e. Family.
   f. Tribe.
   g. Genus.
   (6-5, 6)

4. *a.* *Bacillus* – genus.
   b. *subtilis*–species.
   c. *niger*–variety.
   (6-7-12)

5. First, you would gather all available data about the organism such as morphology, behavior on laboratory culture, antigenic makeup, and other specific facts. Then, correlate this information with data of "type species"–morphologically similar genera. This would usually allow you to decide what genus your isolate belongs in. If there is still doubt as to the identity of the unknown, you should work with a reference laboratory to double check your data. The finding of a previously unknown bacterium in the clinical laboratory is a rarity. (6-9)

6. A strain or variety. (6-12)

7. Family. (6-13)

8. Morphological characteristics include size, shape, and arrangement of cells and internal cellular structures. (6-18)

9. Spherical–coccus, rod-shaped–bacillus, spiral-shaped–spirillum. (Table 4)

10. Autotrophic, heterotrophic, heterotrophs. (6-20-22)

11. Micron. (7-2)

12. Nucleus. (Fig. 15; Table 5)

13. Peritrichous. (Fig. 17)

14. Palisade formation (7-4; Table 6; Fig. 17)

15. *Gaffkya, Sarcina.* (7-6; Table 6)

16. *a.* Occurs singly.
    b. Occurs in pairs attached end to end.
    c. Occurs in chains attached end to end.
    d. Occurs as rows of bacilli – side by side. (Table 6)

17. *a.* Mutation. Change within the genetic structure of cells in a culture.
    b. Adaptation. Change in appearance or behavior influenced by environmental factors. (7-9)

18. Spontaneous mutation. (7-10)

19. First, you would see that a vast number of bacteria that were plated did not grow. In fact, you might observe slow growth on the plate. In time, however, you would see isolated colonies of the resistant bacteria. This exact phenomenon is seen in-vivo also. The patient may be seen to improve after treatment and then relapse due to the increased growth of a resistant strain. (7-10)

20. Adaptation. (7-11)

21. A variation in the naturally occuring size and shape of a particular bacterium. (7-13)

22. *a.* Nutrition (proper food).
    b. Moisture.
    c. Acidity/alkalinity (pH).
    d. Temperature.
    e. Gas exchange.
    (8-3)

23. Mesophilic. (8-7)

24. An organism which prefers to grow as an anaerobe, but can adapt to aerobic conditions. The vast majority of medically significant bacteria are in this category. (8-12)

25. An organism for which oxygen is toxic, except in minute amounts. (8-14)

26. Shape, outline, size, and texture. (8-15)

27. Spread out. (8-17)

28. Texture. (8-21)

29. Mucoid. (8-21)

30. An S-type colony is smooth, translucent, convex, glistening, and circular in appearance. An R-type colony is generally dull, less translucent than an S-type colony, and has an irregular edge with a wrinkled or rough, granular surface. (8-22, 23)

31. Very definitely! Colonies on dye-free media are white or gray and opaque or translucent. However, pigment-producing organisms on different dye containing media will produce color, depending upon the specific media. From your own experience, you know this is true in enteric work. For instance, an *E. coli* will show different colony colors when plated on EMB agar–green-gold metallic sheen, MacConkey Agar–pink to red, and blood agar–gray white. (8-24)

32. Leukobases, lipochromes. (8-25)

33. Brewer anaerobic jar, Pyrogallol anaerobic technique (Bray dish or filter paper), and thioglycollate medium. (8-28, 30-35)

34. 2 to 3 percent. (8-29)

35. Any culture medium should have sources of organic or inorganic carbon, nitrogen, and inorganic salts. But other substances–blood, serum, amino acids, or vitamins–may also be necessary. (9-3)

36. Carbohydrates are detrimental in a culture medium when we are trying to study formation of hemolysins. (9-4)

37. 1.5 to 2.0 percent. (9-5)

38. Basal, enriched, isolation, and biochemical. Isolation media may be either selective or differential in their actions. (9-9)

39. A basal medium contains only basic ingredients needed for the growth of nonfastidious organisms. An enriched medium is a basal medium which has certain enrichment materials added, as needed, to grow specific fastidious organisms. Isolation media are basal media to which specific (usually inhibitory) agents have been added as an aid to obtaining a pure culture of an organism from a mixed population. (9-9-12)

40. Differential media distinguish between closely related bacteria on the basis of fermentation reactions or the production of metabolites whose presence can be detected by a color change in the medium. Selective media contain an additive which permits one bacterial species to grow while simultaneously inhibiting the reproduction of contaminating organisms. (9-13, 14)

41. Because water which has been stored for a long time absorbs enough gases from the air during storage to alter the pH of the finished medium; therefore, it is best to use *fresh distilled* water. (9-17)

42. *a.* (3).
    b. (1).
    c. (4).
    d. (2).
    e. (7).
    f. (5).
    (9-17-24)

43. *a.* Bubbles tend to "spring" wire loops, thereby offering a chance for bacteria to become airborne.
    b. Media surfaces may be cut by the loop, and in so doing, be wiped free of most bacteria. (9-27-29)

44. The agar base was allowed to cool down too much before adding blood and pouring into plates. A lumpy medium does not have an even distribution of nutrients and enrichment materials, thereby giving poor culture results and erroneous hemolytic reactions. The same organism may give us several different reactions on the same plate. It is also hard to streak a plate with lumpy surfaces. (9-31)

45. Absolutely never! All plates should be inverted and incubated at least 18 hours to check for sterility. Otherwise, we cannot be sure that airborne contaminants have not been deposited on the medium surface. The finest sterile technique cannot preclude this eventuality. (9-37)

46. No! Excessive moisture enhances the possibility of contamination yourself and the entire laboratory during plate streaking. Moreover, a film of water or water droplets makes it difficult to obtain isolated colonies of bacteria. (9-37)

47. *a.* Inhibits the action of penicillin and streptomycin in specimens from patients who are taking these drugs.
    b. Has bacteriostatic action and can be effectively used to inhibit staphylococci when we are trying to isolate streptococci.
    c. This bile salt inhibits growth of Gram-positive bacteria. It will also suppress the motility of flagellated bacteria.
    d. Retards growth of most Gram-negative bacteria. It is especially useful when trying to isolate *C. diphtheriae.*
    e. Can be used to eliminate contaminating organisms. Especially useful in fungus cultures.
    (9-38-47)

48. The ability of a specific organism to attack and break down a particular carbohydrate is a useful characteristic. By carefully selecting the test carbohydrates, we can obtain a pattern of fermentation reactions which are characteristic of specific organisms. (9-49, 50)

49. Lactose, sucrose, and glucose. (9-51)

50. Indole. (9-55)

51. The MR test measures the ability of an organism to produce acids from dextrose. These acids are not further broken down. Hence, the Methyl Red (MR) indicator registers a change of pH in the culture. The Voges-Proskauer (VP) procedure tests the ability of an organism to produce an acid from dextrose, but the acid (pyruvic) is then converted to a neutral end produce, acetyl-methyl-carbonal. (9-57, 58)

52. Gelatin liquifaction. (9-59)

53. Lactose. (9-62)

CHAPTER 4

1. How to separate pathogenic microbes from harmless organisms with which they grow in close association in nature. As simple as this may sound, this is one of the most perplexing problems in diagnostic bacteriology. (Intro.)

2. As the wire loop is streaked back and forth across the plate, fewer and fewer organisms are deposited on the medium surface. Eventually only single cells are deposited. The single cells can then multiply into well-separated, pure colonies which can be subcultured for identification. (10-3)

3. A mixed culture subcultured to a broth or agar medium will yield a typical results when used in biochemical tests. (10-4, 5)

4. Pour plates are most useful to determine the type of hemolysis produced by strains of streptococci, especially the "O" hemolysin of beta hemolytic Streptococcus. (10-8)

5. Drain it off! If the slant is too wet, resulting growth will not be characteristic of the species. (10-11)

6. *a.* Liquid broth culture: Used to maintain viability of an organism.
   b. Slant culture: Used in biochemical tests as well as to maintain stock cultures in the laboratory.
   c. Stab culture: Also used in biochemical tests especially for anaerobic organisms.
   (10-9-12)

7. It allows for growth of isolated colonies from those specimens which contain a large number of bacteria. Diluting the specimen with a known volume of fluid also permits us to estimate accurately the number of bacteria present in a heavily contaminated specimen. (10-13)

8. The membrane is placed on a plate of conventional nutrient agar. Nutrients necessary to the growth of bacteria are absorbed through the membrane by the growing colony. (10-14)

9. Blood agar aids in detecting hemolysin-producing organisms, differentiates between types of hemolysis, and furnishes the nutrients required by many fastidious pathogenic bacteria. (11-3)

10. There are two acceptable methods: one, make two or three cuts in the heavily inoculated area of the plate, or alternately, place a sterile coverslip on an area of the streaked plate to provide anaerobic conditions on the agar surface under the coverslip. (11-4)

11. To isolate enteric organisms which might be found on occasion in the throat or nasopharyngeal area, especially in young children. (11-9)

12. First, we would set up our routine smears and cultures. Second, we would start our AFB procedures. Processing must be done in this order, because the drastic digestion and concentration techniques for the tubercle bacilli destroy other microorganisms. (11-13)

13. We must always be careful when handling infectious material. A hood is strongly recommended when handling AFB specimens. Also, it helps to dip the loop in phenol before flaming it. This reduces contamination from sputtering as the sputum incinerates. (11-15)

14. Concentration procedures free mycobacteria from the tissue particles in which they may be lodged. The digestion of tissues which takes place during concentration also destroys organisms other than the tubercle bacillus, so that the contaminants won't overgrow the TB germ during incubation on isolation media. (11-16)

15. It allows digestion to proceed slowly on those specimens which cannot be processed right away. It is useful on specimens having large quantities of mucous. It can be used to mail specimens. Tubercle bacilli remain viable after a week's exposure. (11-19)

16. Middlebrook 7H10 agar requires a 2- to 5-percent CO_2 incubator, candle jar, or by sealing the cultures in a plastic bag with a culture of *M. phlei.* (11-24)

17. Heparin. (11-27)

18. *Cryptococcus neoformans.* (11-29)

19. Exudates can contain any of the most commonly isolated microbes pathogenic to man. Therefore, we must know the anatomical site of the body from where the specimen was collected, and the morphology of the organisms as seen on direct smears before we can decide what media to use to culture the specimen. (11-31-33)

20. If 100,000 bacteria or more per milliliter are isolated from a "clean" or "midstream" voided urine, a disease process is indicated. Counts ranging from 10,000 to 100,000 suggest an infection. A count of less than 10,000 per ml. of urine is generally considered insignificant. (11-36)

21. 64 × 10 × 1000 = 640,000 ml. (11-38)

22. Tannic acid (2 to 3 g.) should be added and the entire solution mixed. The mixture should then be placed in the refrigerator overnight (or for 12 hours). A brown precipitate will form which will contain the organism. After centrifugation, the sediment is ready to inoculate to media and prepare smears. (11-41)

23. These broths will inhibit the growth of saprophytes while allowing the pathogens to grow. This allows for the recovery of pathogens when a very few such organisms are present. (11-45)

24. There are a great number of media which you can use to culture stool specimens. Suspicion of what may have caused a patient's condition will give you a better idea of what media to use. Knowing what the clinical symptoms are might help you to decide what genera of organism you are dealing with. Ask the physician–then decide. You may use your usual routine primary media or a specific–highly inhibitory–media, or both. Besides the use of an enrichment broth, primary plating some of the original specimen will save time if you find suspected organisms on the plates as isolated colonies. These colonies can then be picked and restreaked for purification and confirmatory culture steps. (11-46-49)

25. For the cultivation of *Streptococcus faecalis* organisms. (11-51)

26. You can overcome the bacteriostatic effect of a drug by (1) limiting the inoculum to less than 5 percent of the total culture fluid volume (dilution will lower the drug concentration below an inhibitory level); (2) adding 5 mg. % PABA to counteract sulfonamides; (3) incorporating the enzyme penicillinase to inhibit penicillin or streptomycin; or (4) waiting and hoping that the natural deterioration of a drug during the incubation period will let the bacteria grow. (11-57)

27. This type of smear generally is diagnostic of gonorrhea. You should also culture some of the secretion on blood agar and chocolate agar under CO_2 to confirm the presence of *Neissena gonorrheae.* (11-59; Fig. 40)

28. It is very important to know the origin of the specimen. You should consult with the pathologist or surgeon to insure that you know what organisms might be expected in the specimen based on clinical evidence. The use of thioglycollate medium is certainly important to try to isolate both anaerobes and aerobes. Stained smears are definitely helpful in determining the choice of media or culture conditions. (11-61-63)

29. *a.* Always use clean slides.
    b. Make more than one slide of a specimen.
    c. Air dry the smear completely.
    (12-4)

30. Place a drop of normal saline on the slide, then emulsify the material on the swab and smear the material evenly over the slide surface. (12-7)

31. We fix a slide by passing it through the flame of a bunsen burner two or three times. It should be warm to the touch—not HOT! Heat-fixing makes the bacteria stick to the slide so they don't wash off during staining. Overheating will char the bacteria and alter their staining properties. (12-10)

32. Chromophore. (12-12)

33. Mordant. (12-13)

34. The direct stain stains the microorganisms to give general characteristics of cell morphology, whereas the indirect stain merely colors the background leaving the organism unstained and contrasted against the background. (12-14)

35. Selective stain. (12-14)

36. It means that cellular constituents are concentrated at both ends of the cells and stain more intensely or accumulate dye to a greater extent. Bipolar staining shows that the organism is characterized by a concentration of cellular elements at both ends of the cell—elements that stain more intensely than the cytoplasm. (12-15)

37. Concentration of dye. the concentration of the bacteria on the slide, and the length of time that the stain is in contact with the bacteria. (12-16)

38. Within the ribonucleic acid component of the cytoplasmic membrane. (12-19)

39. Gram-negative. (12-21)

40. Smears were not properly air-dried before heat-fixing and the debris is a result of precipitated protein material carried over from the culture or specimen. (12-22)

41. The phenol serves as a carrier of the fuchsin dye in penetrating the lipid layer of the cell. (12-24)

42. The Ziehl-Neelsen stain uses heat to force the stain into the cell, whereas the Kinyoun method uses a surface tension reductant such as tergitol. (12-25)

43. The finer the carbon particles in India ink, the better the ink is for the negative staining procedure. (12-26)

44. By adding phenol in a 0.5-percent concentration by volume to the India ink. (12-26)

45. The serum or plasma causes the capsule to swell and allows easier penetration of the dye. (12-27)

46. The dye covered smear is steamed and the heat drives the stain into the spore coat. (12-28)

47. To observe motility of living microorganisms. (12-29)

48. Flagellar motion is generally directional movement in which the individual cell moves from one place to another. Brownian movement is a vibratory motion of a bacterial cell in a limited fixed position caused by molecular bombardment. (12-29)

CHAPTER 5

1. Antibiotic. (13-1)

2. The term "selective toxicity" describes a concept of destroying or suppressing the growth of pathogenic microbes by antimicrobial agents that do not injure the tissues of the human host. (13-3)

3. (a) Cell wall formation, (b) cytoplasmic membrane function, (c) protein synthesis, (d) nucleic acid metabolism, and (e) intermediary metabolism. (13-7)

4. Bacteriostatic. (13-9)

5. Mucopeptides. (14-2)

6. The peptide linkage between amino acids permits the union of these acids to form larger molecules of nitrogenous substances such as polypeptides and proteins. (14-3)

7. Higher. (14-4)

8. Cycloserine molecules resemble molecules of the amino acid alanine. The bacterial cell, not able to tell the difference, picks up the cycloserine molecule. It does not function as well as the alanine molecule. Therefore, cell peptide formation and possibly other reactions involving alanine, are blocked. (14-6)

9. They differ in the chemical composition of their side chains. (14-7)

10. It provides a barrier that regulates the flow of fluids and metabolites into and out of the cell. It may also aid in the synthesis of other cell structures. (14-9)

11. The antimicrobial agent combines with a vital part of the membrane, thereby altering the membrane's ability to move material into and out of the cell. There may also be a loss of selective permeability such that some of the internal components can leak out of the cell. (14-10)

12. The fungi have a sterol component in their cytoplasmic membrane with which the antimicrobial agent can act. The bacteria do not. (14-12)

13. Soluble RNA serves as a transport medium between raw materials (amino acids) and the protein manufacturing site of the cell. (14-18)

14. Messenger RNA transmits from the nuclear DNA to the ribosome, the necessary genetic coding instructions for making protein. (14-19)

15. Anabolism. (14-24)

16. *b.* PSA, and *c.* sulfonamide. (14-27)

17. The cells produce an enzyme, penicillinase, that breaks down penicillin. (15-1)

18. In many species drug tolerance can be transferred between cells. (15-2)

19. Spontaneous mutation and the exchange of genetic material between cells. (15-3)

20. Transformation is the process whereby one cell takes up the nuclear elements (genes) derived from another. Transduction is the transfer of genetic material from one cell to another with the help of bacteriophages (viruses). (15-4, 5)

21. With conjugation, genetic material is passed directly from one cell to another during physical contact. With transformation and transduction, the transfer of material is indirect, not requiring that two cells have physical union. (15-4-6)

22. Dilution and diffusion of the antimicrobial agent. (16-3)

23. Dilution. (16-6)

24. *a.* A distinct end point.
    b. Applicable to all therapeutic drugs.
    c. Suitable for use with all pathogens.
    d. Give results in a short time.
    e. Permit recognition of pathogens as opposed to contaminants.
    f. Differentiate strains which are morphologically similar.
    (16-8)

25. *a.* pH of media.
    b. Composition of media.
    c. Size and nature of inoculum.
    d. Incubation time.
    e. Growth requirement differences.
    f. Drug stability.
    (16-9)

26. 0.5. (16-11)

27. A heavy inoculum may provide enough organisms to neutralize the antibiotic and overgrow a zone of inhibition, thus giving a false-negative sensitivity reaction. (16-12)

28. *a.* As soon as colonies appear on plating media macroscopically they can be subcultured for sensitivity testing before positive identification is made.
    b. Sensitivity discs can be applied directly to a plate streaked with the original clinical specimens.
    (16-15, 16)

29. After 24 hours, growth may be so great as to obscure some sensitivity results. (16-17)

STOP– 1. MATCH ANSWER SHEET TO THIS EXERCISE NUMBER. 2. USE NUMBER 1 PENCIL.

90412 01 21

VOLUME REVIEW EXERCISE

Carefully read the following:

DO'S

1. Check the "course," "volume," and "form" numbers from the answer sheet address tab against the "VRE answer sheet identification number" in the righthand column of the shipping list. If numbers do not match, take action to return the answer sheet and the shipping list to ECI immediately with a note of explanation.
2. Note that numerical sequence on answer sheet alternates across from column to column.
3. Use only medium sharp # 1 black lead pencil for marking answer sheet.
4. Circle the correct answer in this test booklet. After you are sure of your answers, transfer them to the answer sheet. If you *have* to change an answer on the answer sheet, be sure that the erasure is complete. Use a clean eraser. But try to avoid any erasure on the answer sheet if at all possible.
5. Take action to return entire answer sheet to ECI.
6. Keep Volume Review Exercise booklet for review and reference.
7. If *mandatorily* enrolled student, process questions or comments through your unit trainer or OJT supervisor.
   If *voluntarily* enrolled student, send questions or comments to ECI on ECI Form 17.

DON'T

1. Don't use answer sheets other than one furnished specifically for each review exercise.
2. Don't mark on the answer sheet except to fill in marking blocks. Double marks or excessive markings which overflow marking blocks will register as errors.
3. Don't fold, spindle, staple, tape, or mutilate the answer sheet.
4. Don't use ink or any marking other than with a # 1 black lead pencil.

Note: The 3-digit number in parenthesis immediately following each item number in this Volume Review Exercise represents a Guide Number in the Study Reference Guide which in turn indicates the area of the text where the answer to that item can be found. For proper use of these Guide Numbers in assisting you with your Volume Review Exercise, read carefully the instructions in the heading of the Study Reference Guide.

162

Multiple Choice

Note: The first three items in this exercise are based on instructions that were included with your course materials. The correctness or incorrectness of your answers to these items will be reflected in your total score. There are no Study Reference Guide subject-area numbers for these first three items.

1. If I tape, staple or mutilate my answer sheet; or if I do not cleanly erase when I make changes on the sheet; or if I write over the numbers and symbols along the top margin of the sheet,

   a. my answer sheet will be unscored or scored incorrectly.
   b. I will be required to retake the VRE.
   c. my answer sheet will be hand-graded.
   d. I will receive a new answer sheet.

2. So that the electronic scanner can properly score my answer sheet, I must mark my answers with a

   a. pen with blue ink.
   b. number 1 black lead pencil.
   c. ball point or liquid-lead pen.
   d. pen with black ink.

3. The form number of this VRE must match

   a. my course number.
   b. the number of the Shipping List.
   c. the form number on the answer sheet.
   d. my course volume number.

Chapter 1

4. (100) Who is known as the father of medicine?

   a. Redi.
   b. Pasteur.
   c. Fracastoro.
   d. Hippocrates.

5. (100) The theory that living organisms could originate from nonliving materials is known as the theory of

   a. spontaneous generation.
   b. organismic multiplication.
   c. microfermentation.
   d. self-multiplication.

6. (100) The father of aseptic surgery is

   a. Louis Pasteur.
   b. Robert Koch.
   c. Joseph Lister.
   d. Francesco Redi.

Chapter 2

7. (101) The Standard Form (SF 514 series)

   a. can be signed by authorized ward personnel if the physician has signed the DD Form 728.
   b. can be completed if the nurse thinks the particular test is necessary.
   c. need not be signed by the physician if he has signed the Doctor's Orders sheet.
   d. must be signed by the physician.

8. (101) Material that has passed through the walls of a vessel and into surrounding tissues is called

   a. a transudate.
   b. a serous fluid.
   c. an infusion.
   d. an exudate.

177

9. (101) Which of the following is particularly difficult to isolate if there is a delay in culturing rectal swabs?

a. *Shigella* spp.
b. *Proteus morganii.*
c. *E. coli.*
d. Staphylococcus *spp.*

10. (101) Why are catheterized specimens obtained for urine cultures?

a. Voided specimens may contaminate the collection bottle.
b. Catheterizing the patient is more convenient than collecting a "clean catch."
c. Voided specimens may contain saprophytes.
d. Cultures can be set up more easily with a catheterized specimen.

11. (101) The purpose of the midstream urine analysis is to help identify infection of the

a. urethra.
b. bladder.
c. ureters.
d. kidney.

12. (102) If a body fluid is sent to the laboratory for bacteriological testing and it has clotted, you should

a. discard the specimen and ask for a repeat.
b. culture the supernatant fluid.
c. culture the clot.
d. add an anticoagulant.

13. (102) When collecting specimens for viral examination, what name is applied to that specimen collected during the active phase of the disease?

a. Convalescent specimen.
b. Acute specimen.
c. Immediate specimen.
d. Viral specimen.

14. (102) Because of the danger of personnel getting an infection, laboratory technicians should refuse to accept a specimen for bacteriological examination if

a. there is no request slip.
b. it does not have a tight-fitting lid.
c. the specimen is not wrapped.
d. there is evidence of a contaminated outer surface.

15. (102) A laboratory technician is performing a bacteriological examination of the contents of a diaper. To prevent self-contamination, he should

a. make certain the specimen is fresh.
b. *not* rinse the diaper.
c. work only on an absorbent surface.
d. refuse the work if the request form mentions enteric bacteria.

16. (102) Desiccation is defined as the

a. process whereby metabolic activities are slowed down to prevent further multiplication.
b. addition of water to rehydrate dried bacteria.
c. preservation of bacteria by quick freezing.
d. removal of water by drying.

17. (102) The initial act of first aid for a laceration of the finger due to handling broken contaminated glassware is to

a. proceed to the treatment room to see a physician.
b. determine what organism was on the glassware.
c. cleanse the wound with running water.
d. stop the flow of blood.

18. (103) The major difference between a disinfectant and an antiseptic is that

a. antiseptics inhibit growth; disinfectants kill the bacterial vegetative cell.
b. disinfectants inhibit growth; antiseptics kill the bacteria.
c. disinfectants have a lesser degree of lethal action against bacteria.
d. antiseptics completely destroy or remove all forms of a microorganism, including the spore forms.

19. (103) In the growth curve of microorganisms, the number of viable organisms reaches its maximum in the

a. lag phase.
b. logarithmic phase.
c. senescent phase.
d. stationary phase.

20. (103) A slightly increased temperature generally

a. increases the disinfectant's viscosity.
b. increases a disinfectant's effectiveness.
c. increases the bacterial surface tension.
d. decreases the acidity of the bacteria's surroundings.

21. (104) If blood serum is to be sterilized, which of the following devices would be the best to use?

a. The Arnold Sterilizer.
b. The autoclave.
c. A gauze-cotton filter.
d. The inspissator.

22. (104) Boiling, 24-hour incubation, and reboiling is the principle used in

a. home preservation of foods.
b. the Arnold Sterilizer.
c. the Seitz filtration system.
d. the autoclave.

23. (104) Which of the following filtering techniques uses an asbestos pad?

a. Berkfield.
b. Chamberland.
c. Pyrex fritted glass.
d. Seitz.

24. (104) The sterilizing effect of filters is achieved mostly by

a. sieve action.
b. chemical action.
c. absorption.
d. mechanical destruction.

25. (105) The sulfonamide drugs are specific chemical combinations that are effective chemical sterilizing agents because they

a. prevent the microorganism from receiving sufficient nutritional substances.
b. are lethal to living cells due to protein coagulation.
c. enter the cell and inhibit enzyme activity.
d. combine indiscriminately with compounds within a bacterial cell.

26. (105) Chlorine is a useful disinfectant because it has high bactericidal properties and because it

a. is nonpoisonous.
b. does not evaporate easily.
c. spreads uniformly over all surfaces.
d. effectively disinfects water.

27. (105) A substance (or substances) often used as a surgical soap and hand wash is

a. beta-propiolactone.
b. lysol and creolin.
c. bis-phenols combined with suitable surfactants or detergents.
d. pure phenol diluted with ethyl alcohol.

28. (105) The sterilizing action of a synthetic detergent is *not* decreased by

a. distilled water.
b. saline water.
c. organic matter.
d. random mixing of different types of detergents.

29. (105) Detergents that have a water-soluble group of particles which dissociate to form positively charged ions are known as

a. anionic detergents.
b. nonionic detergents.
c. cationic detergents.
d. natural detergents.

30. (106) Which of the following organisms is usually used for determining sterility by the autoclave method?

a. *Bacillus stearothermophilus.*
b. *Bacillus subtilis.*
c. *Bacillus subtilis (globigii).*
d. *Clostridium tetani.*

31. (106) From the information furnished, determine the phenol coefficient. Phenol at a dilution of 1:100 killed an organism in 10 minutes. The unknown disinfectant killed the organism in 10 minutes but at a dilution of 1:200.

a. 1.0.
b. 2.00.
c. .5.
d. .2.

32. (106) Laboratory procedures in clinical bacteriology are found in

a. AFM 160-53.
b. AFM 160-52.
c. AFM 160-48.
d. AFM 160-28.

Chapter 3

33. (107) Organisms that obtain energy from organic carbon sources are known as

a. autotrophic bacteria.
b. saprophytic bacteria.
c. heterotrophic bacteria.
d. nonpathogenic bacteria.

34. (107) In classifying bacteria, which of the following represents a family name?

a. Thiorhadaceae.
b. Eschericheae.
c. Pseudomonadales.
d. Rhodobacteriineae.

35. (107) Organisms that possess enough similar qualities to be grouped as species but that differ sufficiently to be considered as a separate group within the species are referred to as

a. varieties.
b. families.
c. subtribes.
d. genera.

36. (108) Abnormal, bizarre shapes assumed by bacteria in aging cultures are referred to as

a. contaminants.
b. involution forms.
c. reproduction forms.
d. plemorphic forms.

37. (108) A micron is

a. 2.54 inches.
b. 1000 mm.
c. 1/20000 of an inch.
d. 1/1000 mm.

38. (108) An Angstrom unit is equal to

a. 1/10000 of a mm.
b. 1/1000000 of a mm.
c. 10^{-7} microns.
d. 10^{-7} mm.

39. (109) Bacteria that grow best at a temperature of 15° to 26° C. are called

a. psychrophilic.
b. mesophilic.
c. pleomorphic.
d. thermophilic.

40. (109) H-type colonies of *Proteus* spp. are due to

a. capsular material.
b. flagellar motion.
c. Ohne-hauch forms.
d. urease production.

41. (109) A transformation from a "smooth" to a "rough" colony is usually the result of

a. the loss of the bacterial reproduction process.
b. the loss of the bacterial slime layer or capsule.
c. incubation of the culture at an elevated temperature.
d. gene transfer.

42. (109) If some chemical substance other than free oxygen serves as the hydrogen acceptor, the respiration is said to be

a. eurobic.
b. aerobic.
c. anaerobic.
d. obligate.

43. (109) What substance is present in most media to serve as an available source of carbon and nitrogen?

a. Mannitol-salt.
b. Carbohydrates.
c. Agar.
d. Peptone.

44. (109) Compounds that have been excreted by bacteria and oxidized to form a colored product are pigments known as

a. leukobases.
b. lipochromes.
c. opaque pigments.
d. reduced pigments.

45. (109) Free oxygen is toxic to which of the following?

a. Microaerophilic bacteria.
b. Faculative anaerobes.
c. Obligate anaerobes.
d. Obligate aerobes.

46. (110) What two reagents are used in the Bray dish technique for obtaining 10 percent CO_2?

a. Pyrogallic acid and sulfuric acid.
b. Pyrogallic acid and sodium carbonate.
c. Pyrogallic acid and sodium bicarbonate.
d. Sulfuric acid and sodium bicarbonate.

47. (110) Candles should be placed high in the candle jar because

a. if placed low, the flame will be extinguished by CO_2 before the desired 2 to 3 percent concentration is reached.
b. CO_2 rises and in so doing increases the O_2 content.
c. it is easier to light the candle before sealing the lid.
d. moisture accumulating in the lower part of the jar might extinguish the flame too early.

48. (111) The most common means of sterilizing media is by

a. ultraviolet irradiation.
b. Seitz filtration.
c. membrane filtration.
d. autoclave sterilization.

49. (111) What percent agar is generally used in preparing a solid medium?

a. 1.5 to 2.0.
b. 3.0 to 5.0.
c. 5.5 to 7.0.
d. 7.5 to 9.0.

50. (111) Urease medium is used to detect urease production and is an example of

a. a selective medium.
b. an enzyme medium.
c. a catalase medium.
d. a biochemical medium.

51. (111) A premixed, dehydrated lactose broth should not be overheated because heat causes the lactose to

a. precipitate.
b. decompose.
c. gel.
d. liquefy.

52. (111) The primary reason for using a solid medium is to

a. reduce contamination.
b. prevent contaminants.
c. produce pure colonies.
d. produce the best growth.

53. (111) An agar, such as eosin-methylene blue, on which enteric pathogens are colorless and common coliforms are dark purple is classed as

a. a selective medium.
b. an isolation medium.
c. an enriched medium.
d. a differential medium.

54. (111) Enriching a basal medium makes it suitable for culturing

a. pathogens.
b. nonpathogens.
c. aerobes.
d. anaerobes.

168

55. (111) A basal medium is used to culture which of the following organisms?

a. Nonpathogenic. c. Nonfastidious.
b. Pathogenic. d. Anaerobic.

56. (112) The correct ratio of butt length to slant length in tubed media for enteric cultures is

a. 1:1. c. 1:3.
b. 1:2. d. 2:1.

57. (112) *Fresh* distilled water should be used in rehydrating powdered culture media because fresh water

a. has not had time to absorb gases which could alter the final pH.
b. has not had time to become contaminated.
c. is more alkaline in pH than stale water.
d. does not have to be filter sterilized.

58. (112) Blood should be added to sterilized media after the media have cooled to

a. 50° C. to 60° C. c. 35° C. to 45° C.
b. 45° C. to 55° C. d. 25° C. to 35° C.

59. (112) The best technique for dispensing repeatedly consistent volumes of a medium is to use

a. a funnel. c. a graduated cylinder.
b. a rubber tube and pinch clamp. d. an automatic syringe.

60. (113) Sodium thioglycollate, in addition to its reducing action, also neutralizes which of the following?

a. Para-aminobenzoic acid. c. Mercurial and silver disinfectants.
b. Sulfonamide. d. Bacteria.

61. (113) Triple sugar iron agar (TSI) differs from Kliger's iron agar (KIA) in that TSI

a. shows H_2S production; KIA does not.
b. contains sucrose; KIA does not.
c. is usually slanted; KIA is not.
d. is used for differentiating enteric organism; KIA is not.

62. (113) Liquefaction of egg albumin is an example of

a. gelatin liquefaction. c. proteolysis.
b. proten denaturation. d. protein fermentation.

63. (113) The production of acetyl-methyl-carbinol will give a positive

a. TSI slant. c. Ninhydrin reaction.
b. methyl red test. d. Voges-Proskauer test.

Chapter 4

64. (113) To prevent the swarming of *Proteus* spp., you can add which of the following to the media?

a. Agar. c. Chloral hydrate.
b. 5 percent whole blood. d. Sodium desoxycholate.

183

65. (114) To be sure you are picking a "pure" colony for further identification, you should

a. pick from the center of the colony.
b. gather up the whole colony.
c. gather several colonies that look alike.
d. put the loop under the colony and remove the media and the colony.

66. (114) When performing the blood agar *pour plate* technique for the isolation of bacteria, you should add the blood at

a. 80° C.
b. 48° C.
c. 37° C.
d. 25° C.

67. (115) Characteristic pigmented colonies of *Corynebacterium diphtheriae* on potassium tellurite agar usually develop in

a. 12 hours.
b. 18 hours.
c. 24 hours.
d. 48 hours.

68. (115) Thioglycollate broth tubes are heated in a water bath before use to

a. drive off dissolved oxygen.
b. dissolve the thioglycollate.
c. activate the indicator.
d. sterilize the broth.

69. (115) You should make two or three cuts in the blood agar when streaking a throat swab to

a. help clean the loop.
b. help detect "O" hemolysins.
c. test the texture of the media.
d. let air in, which facilitates dextrose fermentation.

70. (116) Which of the following are you least likely to isolate from a thioglycollate broth tube?

a. *Streptococcus* spp.
b. *Staphylococcus aureus.*
c. *Klebsiella pneumoniae.*
d. *Mycobacterium tuberculosis.*

71. (116) The main advantage of the NAC concentration tech [illegible] the NaOH or TSP method is that

a. with NAC a higher concentration of alkali is used to dissolve the mucus.
b. the sediment from the NAC concentration is easier to stain.
c. with NAC there is a greater survival of the tubercule bacilli during digestion.
d. the NAC solution is less expensive than NaOH or TSP.

72. (116) If we expect to find *M. tuberculosis* organisms on a direct Ziehl-Neelsen smear, there should be at least how many acid-fast organisms present in the sputum?

a. 100,000 AFB per ml.
b. 100,000 AFB per specimen.
c. 100,000 AFB per cu. mm.
d. 1,000,000 AFB per ml.

73. (117) Cycloheximide is added to media to inhibit

a. only bacteria in fungus cultures.
b. only saprophytic molds from fungus cultures.
c. both bacteria and saprophytic molds in fungus cultures.
d. yeast forms of fungus.

74. (116) In the sodium hydroxide concentration technique for acid-fast bacilli, the solution is titrated to a neutral endpoint using

a. hydrochloric acid solution.
b. sodium hydroxide solution.
c. trisodium phosphate.
d. normal saline.

75. (118) The first step in isolating and identifying stool pathogens is

a. primary plating.
b. enrichment.
c. serological identification.
d. Gram staining.

76. (118) A regular colony count on a patient with a urinary tuberculosis infection is likely to show

a. no tubercule bacilli.
b. a few tubercule bacilli.
c. many tubercule bacilli.
d. TB—too numerous to count.

77. (118) If you received a stool specimen on a patient suspected of having typhoid fever, which of the following plating media should you use?

a. S-S agar.
b. EMB agar.
c. Brilliant green agar.
d. Bismuth sulfite agar.

78. (119) Bacteria present in the blood stream are best described by the term

a. infection.
b. bacteremia.
c. anuria.
d. bacteriuria.

79. (118) On doing a urine colony count, a diseased state is indicated when the colony count is

a. less than 10,000 organisms per ml.
b. from 10,000 to 100,000 organisms per ml.
c. over 100,000 organisms per ml.
d. larger than 100,000 organisms per 24-hour specimen.

80. (119) Gonorrhea is most often diagnosed by

a. fermentation studies.
b. plating an agar.
c. biochemical studies.
d. Gram-stained smear.

81. (118) If, while using the dilution plating technique, your undiluted specimen showed 950 colonies on the plate, you would expect to find how many colonies on the 1:100 dilution plate?

a. 95.
b. 9.5.
c. 6.5.
d. 2.

82. (119) The best medium for initially isolating bacteria from tissue specimens is

a. thioglycoleate broth.
b. enriched nutrient broth.
c. blood agar.
d. chocolate agar.

83. (120) The simplest method for detecting motility is

a. a hanging drop smear.
b. to Gram stain the smear.
c. to coverslip the smear.
d. a supravital stained smear.

84. (120) If you prepare a smear by emulsifying a colony in a drop of water, you should be sure that the water is

a. heated to a boil.
b. chemically pure.
c. freshly distilled.
d. bacteria free.

85. (119) Blood cultures should be kept how many days before the culture is reported negative?

a. 2.
b. 14.
c. 21.
d. 28.

86. (120) The color pattern of a stain is determined by the concentration of bacteria, the concentration of dye, and the

a. size of the bacteria.
b. length of the bacteria.
c. pathogenicity of the bacteria.
d. staining time.

87. (120) A basic dye used in staining is technically referred to as

a. a cationic chromophore.
b. an anionic chromophore.
c. a selective stain.
d. a mordant.

88. (120) Very thick smears give false Gram-positive results because they are difficult to

a. stain.
b. decolorize.
c. counterstain.
d. examine.

89. (120) Penetration of basic dye into organisms containing large amounts of lipids or waxes can be facilitated with

a. acid-alcohol.
b. alcohol.
c. iodine.
d. phenol.

90. (120) Which of the following is good for negative staining of bacteria?

a. Ziehl-Neelsen stain.
b. Gram stain.
c. Hiss stain.
d. India ink.

Chapter 5

91. (121) Chemical substances produced by living organisms that suppress the growth of bacteria are referred to as

a. sulfonamides.
b. toxins.
c. antitoxins.
d antibiotics.

92. (121) If an antimicrobial substance inhibits the growth of bacteria, yet does not kill them, this substance can be said to have what effect on the bacteria?

a. Lethal.
b. Bactericidal.
c. Bacteriostatic.
d. Interactive.

93. (122) A "competitive analog" type of antibiotic such as cycloserine is effective because it

a. resembles a natural compound in molecular configuration.
b. can be fitted into the peptide portion of the bacterial building blocks.
c. causes the amino acid sequence within the cell to become normal.
d. is made synthetically.

172

94. (122) The basic structure in protein synthesis in bacteria is

a. ribosomes.
b. polyribosomes.
c. amino acids.
d. peptides.

95. (122) The genetic pattern to be followed in protein synthesis is originated by the

a. messenger RNA.
b. soluble RNA.
c. nuclear DNA.
d. cellular RNA.

96. (122) Gramicidins and tyrocidins interfere with cell

a. permeability.
b. reproduction.
c. respiration.
d. sterols.

97. (122) The genetic substance that determines the nature of a bacterial cell's metabolism is

a. ribonucleic acid.
b. deoxyribonucleic acid.
c. nucleic acid.
d. phospholipide.

98. (122) A loss of selective permeability of the cytoplasmic membrane will result in

a. an antibiotic becoming ineffective.
b. essential cell constituents leaking out.
c. an increased transportation of materials into and out of the cell.
d. increased reproduction.

99. (122) Within the bacterial cell, the breakdown of complex nutrients to simplified usable forms is referred to as

a. respiration.
b. anabolism.
c. catabolism.
d. nucleic acid metabolism.

100. (122) Pseudome tabolites, such as the sulfonamides, are taken up by cells, and this interferes with

a. para-amino salicylic acid metabolism.
b. para-aminobenzoic acid metabolism.
c. ribonucleic acid metabolism.
d. genetic coding.

101. (122) Polymyxin antibiotics destroy bacteria by interfering with cell

a. reproduction.
b. inflow.
c. permeability.
d. outflow.

102. (123) The uptake from the medium by one cell of nuclear elements (genes) derived from another cell is referred to as

a. DNA extraction.
b. transformation.
c. transduction.
d. conjugation.

103. (123) Mutation to drug tolerance is generally considered to be characteristic of the pathogenic bacterial

a. capsule.
b. flagella.
c. ribosome.
d. gene.

187

104. (122) Bacteria are resistant to the antifungal agent Amphotericin B because

a. the bacteria lack the sterols with which the antibiotic combines.
b. the bacteria have sterols with which the antibiotic can combine.
c. bacteria possess a cell membrane.
d. bacteria lack a cell wall.

105. (123) The process in which genetic material is passed during direct cell contact is referred to as

a. replicating viruses.
b. lysogenesis.
c. tranduction.
d. conjugation.

106. (124) A broth is ready to be used in sensitivity studies when there is a

a. slight sediment.
b. visible turbidity.
c. heavy growth.
d. slightly visible precipitate.

107. (124) An antibiotic assay determines

a. drug concentration in a specimen.
b. microbial sensitivity.
c. drug activity.
d. drug effectiveness.

108. (124) Which of the following is a *true* statement concerning antibiotic sensitivity tests?

a. pH has no effect on antibiotic sensitivity tests.
b. All media used as plate media are suitable for the diffusion method of antibiotic sensitivity tests.
c. The presence of serum albumin has no effect on sensitivity testing.
d. Glucose present in a medium may be detrimental to some sensitivity tests.

109. (124) Sensitivity studies for fastidious organisms should be read within

a. 48 hours.
b. 24 hours.
c. 12 hours.
d. 6 hours.

90412 02 0370 0973

CDC 90412

MEDICAL LABORATORY TECHNICIAN—MICROBIOLOGY

(AFSC 90470)

Volume 2

Laboratory Procedures in Clinical Bacteriology
(Part II)

10-3

Extension Course Institute

Air University

Preface

THIS SECOND volume of Course 90412, *Medical Laboratory Technician*, is a continuation of the laboratory procedures in clinical bacteriology which you began studying in Volume 1. It contains information on Gram-positive and Gram-negative organisms along with information on enteric organisms, acid-fast bacilli, and spirochetes.

Printed and bound as a separate inclosure to this volume are foldouts 1–8. Whenever you are referred to one of these foldouts, please turn to the separate inclosure and locate it. Some of these foldouts are in color. You should find the color illustrations most helpful in learning to identify disease-causing organisms.

If you have questions on the accuracy or currency of the subject matter of this text, or recommendations for its improvement, send them to Technical Training Center (MSSTW), Sheppard AFB TX 76311.

If you have questions on course enrollment or administration, or on any of ECI's instructional aids (Your Key to Career Development, Study Reference Guides, Chapter Review Exercises, Volume Review Exercise, and Course Examination), consult your education officer, training officer, or NCO, as appropriate. If he can't answer your questions, send them to ECI, Gunter AFB, Alabama 36114, preferably on ECI Form 17, Student Request for Assistance.

This volume is valued at 36 hours (12 points).

Material in this volume is technically accurate, adequate, and current as of November 1969.

Contents

Page

Preface . . . . *iii*

Chapter

1 THE GRAM-POSITIVE ORGANISMS 1

2 THE GRAM-NEGATIVE COCCI AND COCCOID FORMS 20

3 THE SMALL GRAM-NEGATIVE BACILLI 27

4 THE ENTERIC ORGANISMS 38

5 THE ACID-FAST BACILLI 50

6 THE SPIROCHETES 57

Bibliography 66

CHAPTER 1

The Gram-Positive Organisms

THE FIRST VOLUME of this course was devoted primarily to the fundamentals of bacteriology. We learned something about the nature of bacteria, their processes of growth and reproduction, and their characteristic morphological features. Then we studied the techniques that are commonly used to isolate and cultivate microorganisms. This led to a discussion of staining procedures, biochemical tests, and other means of establishing the identity of microbes isolated from representative clinical specimens. Lastly, we reviewed the action of antimicrobial agents and discussed the principles of sensitivity testing in the clinical laboratory.

2. In this volume we will take up the medically important genera, beginning with the Gram-positive cocci and rod forms that cause some of the most serious human diseases. Then, after describing the Gram-negative cocci and small bacilli or coccoid organisms, we will cover the enteric groups found in the intestinal tract. Finally, we will examine the acid-fast bacilli and the spirochetes. Our study of the pathogens will consist of a brief review of their clinical significance, the general characteristics of these organisms, and accepted steps for identifying them.

3. The Gram-positive bacteria of interest to us are distributed among a dozen or more genera. Seven of these genera contain the coccal forms, and the others consist of rods, or elongated rodlike forms. Collectively, the Gram-positive cocci are responsible for an impressive variety of human infections. These infections range from a relatively simple involvement of the skin and mucous membranes to more serious diseases manifested by pneumonia, septicemia, rheumatic fever, acute glomerulonephritis, or deep tissue abscesses.

4. Among the rod forms we find pathogens that also cause severe superficial and systemic lesions, as well as toxin-producing organisms which give off some of the most poisonous substances known to man. From time to time we will mention the more common saprophytes of various genera as we proceed with a discussion of the Gram-positive organisms, because the saprophytic species complicate the process of isolating and identifying the pathogens.

1. The Staphylococci

1-1. Staphylococci are among the most frequently encountered isolates in the bacteriology laboratory because they are always present in the air, on dust particles, and on the body. Most species are harmless, but almost any one of the saprophytic forms can cause disease if given the right conditions. Ordinarily, however, we think of the pathogenic staphylococci as belonging to one or the other of two species. Their taxonomic relationship is as follows:

Order IV. *Eubacteriales*
  Family VII. *Micrococcaceae*
    Genus II. *Staphylococcus*
      Species. *S. aureus*
        S. epidermidis

1-2. **Clinical Significance.** The staphylococci have been recovered from all areas of the body and from many disease processes. They occur as normal inhabitants on the skin and in the upper respiratory tract of man. Many pathogenic strains are carried in the nasal passages of asymptomatic individuals who transmit the organisms to susceptible persons. These organisms manifest themselves in localized suppurations such as simple pustules or hair follicle infections. In time, the simple infections can develop into carbuncles and even form metastatic abscesses in other tissues. Cases of osteomyelitis, enterocolitis, otitis media, and sinusitis can be of staphylococcal etiology. Pneumonia, meningitis, and endocarditis are relatively infrequent diseases associated with particularly virulent strains. *Staphylococcus aureus* is considered the most highly pathogenic species; *S. epidermidis* is pathogenic to a lesser extent. Infections with *Staphylococcus aureus* often present difficult treatment problems because some strains rapidly develop resistance to certain commonly prescribed antimicrobial agents. From time to time, hospital outbreaks of staphylococcal infections are traced to these resistant forms.

1-3. Staphylococci are also common "culprits" in food poisoning outbreaks. The organisms produce a heat-stable (resistant to boiling) exotoxin while growing in meat products, cream-filled pastries, gravies, potato salad, and similar foods exposed to room temperature for extended periods. Without proper refrigeration, significant amounts of the exotoxin (also called enterotoxin because of its site of action) can be formed in as little as 4 hours. The enterotoxin is rapidly absorbed by the intestinal mucosa, resulting in nausea, vomiting, diarrhea, and acute prostration within 2 to 6 hours after the contaminated food is consumed. Staphylococcal food poisoning is not an infection, but an intoxication resulting from the ingestion of the preformed toxin. Hence, the implicated foods, rather than stool specimens, are more likely to yield the pathogen on bacteriological analysis.

1-4. **General Characteristics.** Members of the genus *Staphylococcus* are spherical or oval, measuring approximately 1 micron in diameter. Most species are typically arranged in irregular clumps resembling a cluster of grapes. In broth cultures single cocci, pairs, and occasional short chains may be seen. These organisms are nonmotile, nonsporeforming, and are characteristically Gram-positive, although Gram-variable cells are frequently observed in smears from old cultures.

1-5. Staphylococci grow well on meat infusion or meat extract media at a temperature of 37° C. Although their optimum growth temperature is around 35° C., they grow readily at any temperature from 15° C. to 40° C. Characteristic colonies grow best under aerobic conditions, but growth takes place in an anaerobic enviornment if hydrogen and uracil are present. Synthetic media used for cultivation must include the two vitamins, thiamin and nicotinic acid. The fresher the medium, the more typical are the colony characteristics. A pH of 7.4 is considered optimum.

1-6. On blood agar, typical colonies of Staphylococcus are generally formed after 18 to 24 hours of incubation at 37° C. The increased carbon dioxide atmosphere provided by a candle jar favors their growth on primary isolation. *Staphylococcus aureus* appear as glistening, circular, orange to white colonies with a butterlike consistency. A clear (beta) hemolysis is normally produced. The colonies of *Staphylococcus epidermidis* are generally circular, smooth, and a pale, translucent white color. The blood agar may or may not be hemolyzed. There are many variations in colony texture and appearance. Occasionally, rough colonies may be overlooked because of their atypical appearance. The roughness of the colony may suggest a fungus or a common soil contaminant. With the staphylococci you may also see mucoid white, mucoid yellow, smooth yellow, smooth white, rough yellow or "G" (gonidial) type colonies. The latter are small (0.5 mm. in diameter) mutant colonies sometimes isolated from septic lesions. These mutants ordinarily do not produce catalase, coagulase, or hemolysins.

1-7. In a nutrient broth culture, staphylococci produce a moderate turbidity with a powderlike sediment in 24 hours at 37° C. When shaken, the sediment swirls upward in the tube and increases the broth's turbidity. Some staphylococci, particularly those that do not produce coagulase, form a thick gelatinous deposit which leaves the supernatant layer clear. If incubated for 48 hours or longer a surface growth, or pellicle, forms.

1-8. **Laboratory Identification.** Special media aid in the identification of *Staphylococcus* species. A medium such as mannitol-salt-agar that contains a high concentration of sodium chloride inhibits most other organisms. Mannitol is incorporated in the medium to detect pathogenic strains, because most pathogens ferment that carbohydrate. The production of acid in the medium causes the phenol red indicator to turn yellow. Mannitol-positive organisms appear as small colonies surrounded by a yellow zone. Mannitol-negative species yield colonies sursurrounded by distinct red or purple zones.

1-9. A number of confirmatory procedures can be performed on colonies of the staphylococci. In foldout 1 we have diagrammed a progression of identification steps based on hemolysis of blood agar and other special tests used to distinguish among the Gram-positive coccus species. Note in foldout 1 that many *Staphylococcus* species produce beta hemolysis, denoted by a clear zone around the colony. Some strains do not hemolyze blood agar under the usual laboratory conditions of cultivation. The reactions of these nonhemolytic strains are shown in the bottom entry of the foldout.

1-10. Formation of the enzyme, catalase, is a key factor in distinguishing between species of Streptococcus and Staphylococcus that produce identical hemolytic patterns on blood agar. When a colony of staphylococci is mixed with hydrogen peroxide, the action of catalase liberates oxygen from the peroxide and gives a vigorous bubbling. The test is negative (no bubbling) in the case of streptococci and mutant forms of staphylococci.

1-11. Coagulase activity is related to pathogenicity of *Staphylococcus* species, but the correlation is not absolute. A fibrin clot is formed in human or rabbit plasma when colonies that produce coagulase are added. The test is not quantitative, and any visible coagulation is a positive test. Coagulation usually occurs within 3 hours. When plasma stands, however, certain labile clotting factors are destroyed. This slows or completely masks the coagulase reaction. Therefore, when aged plasma is used, the test must be observed for at least 24 hours to rule out the chance of a delayed clot which would indicate a positive reaction. Certain staphylococci that produce coagulase also produce staphylokinase (an enzyme which will dissolve the formed clot); the tube of plasma must be checked at 30-minute intervals for presence of a clot in order to prevent the reporting of a false negative reaction.

1-12. **Related Micrococci.** The family Micrococcaceae contains three genera of saprophytes which resemble the staphylococci so closely in morphology that the harmless forms are sometimes mistaken for their pathogenic relatives. Members of the genus *Gaffkya* are found as normal flora in the upper respiratory tract. These Gram-positive, facultative anaerobes, often encapsulated, are ordinarily arranged in groups of four cells—tetrads. Colonies are white or gray, moist, and viscid. *G. tetragenes*, the most common of the group, does not ferment mannitol or produce coagulase.

1-13. The second genus, *Sarcina*, comprises aerobic, Gram-positive cocci that divide in three planes during reproduction. The characteristic "packets" of cells seen on stained smears are usually formed by *Sarcina lutea*, a common contaminant from soil, air, and water. The genus *Micrococcus* holds a heterogeneous group of Gram-positive aerobic cocci which form irregular clumps of cells easily confused with the grape-like clusters of the staphylococci. *Micrococcus* species are coagulase-negative, however, and ferment glucose aerobically. Staphylococci can ferment glucose anaerobically.

1-14. **Phage Typing.** After identifying a pathogenic staphylococcus as to species, the laboratory is sometimes asked to carry out bacteriophage typing. This typing technique affords a means of further identifying strains within a *Staphylococcus* species based on their susceptibility to lysis by different bacteriophages—viruses that attack and destroy bacterial cells. By seeding a plate of medium with a pure culture of the organism and applying droplet suspensions of several known phage types we can establish a pattern of susceptibility/resistance to lysis which serves to "fingerprint" the staphylococcus.

1-15. We simply record the presence or absence of plaques (cleared areas) on the surface of the plate at the site where each phage suspension was deposited. Plaque formation at that site indicates lysis of the cells by phage particles and, hence, susceptibility of the bacterium to that particular phage. There are approximately 22 phages in use today for identifying *Staphylococcus aureus*. These phages are arranged in five groups, each group consisting of one or more specific types.

1-16. In recent years many of the staphylococcal epidemics in hospital surgical and pediatric wards have been traced to organisms lysed by phages of Groups I and III. Strains susceptible to phage types 80 and 81 within Group I have been particularly prevalent in outbreaks of "hospital staph" infections. By periodic typing of staphylococci isolated from the medical staff, patients, and the hospital environment, the bacteriologist can be forewarned of an impending problem with highly virulent, drug-resistant strains.

2. The Streptococci

2-1. The streptococcal forms, although smaller in size than the staphylococci, closely resemble the latter in morphology of the individual cells. On the basis of differences in physiology and chemical makeup, however, the chain-forming cocci are placed in a separate family, the *Lactobacillaceae*. This family includes a variety of pathogenic and saprophytic microbes which tend to grow sparsely on initial isolation in the laboratory.

2-2. Two genera are of interest to the medical bacteriologist—*Streptococcus* and *Peptostreptococcus*. The first of these contains the aerobic and facultatively anaerobic species most commonly implicated in human disease. The peptostreptococci are strict anaerobes or microaerophiles which grow poorly on conventional media. The streptococcal organisms are classified as shown below:

Order IV. *Eubacteriales*
  Family X. *Lactobacillaceae*
    Tribe I. *Streptococceae*
      Genus II. *Streptococcus*
      Genus V. *Peptostreptococcus*

2-3. **Clinical Significance.** A great number of bacterial infections of man are caused by beta hemolytic streptococci. These organisms cause such diseases as puerperal fever, erysipelas, septic sore throat, scarlet fever, impetigo, and acute bacterial endocarditis. Acute glomerulonephritis or rheumatic fever may develop following streptococcal pharyngitis. The alpha hemolytic streptococci that are normal inhabitants of the upper respiratory and intestinal tracts may cause subacute bacterial endocarditis, meningitis, and urinary tract infections. Anaerobes of the genus *Peptostreptococcus* are encountered in pelvic abscesses, the diseased appendix, sinus and ear infections, and in gangrenous wounds. They are capable of giving rise to severe suppurative lesions characterized by an extremely foul odor. The nonhemolytic streptococci are, for the most part, saprophytic forms found naturally in milk and other dairy products. A few strains have been isolated from cases of subacute bacterial endocarditis.

2-4. **General Characteristics.** Streptococci are round or oval and range in size from 0.8 to 1.0 micron. They occur predominantly in chains; however, paired or single cells may also be seen. Characteristic chains are observed more often in smears from broth cultures. The streptococci are nonmotile, nonsporeforming, and Gram-positive. Gram-negative forms are occasionally found in smears from old cultures. Certain *Streptococcus* spp. produce capsules, but the presence or absence of capsules is not a distinctive feature.

2-5. A medium enriched with blood is essential for growth of most pathogenic streptococci. An ordinary meat infusion medium will generally not support growth. The standard blood agars and such broth media as thioglycollate, brain-heart infustion, Todd-Hewitt, and trypticase-soy can be used to cultivate certain species. The majority of streptococci are facultative anaerobes, although some strains of *Peptostreptococcus*, especially those isolated from deep tissue infections, are obligate anaerobes. Growth is best at 37° C. for the pathogenic hemolytic forms. After 24 hours' incubation on the blood agar the streptococci generally appear as small (approximately 1 mm. or less) slightly granular, circular, convex, translucent colonies. Some streptococci give rise to matt or mucoid colonies on primary isolation. After repeated transfer on laboratory media avirulent rough or smooth colonies will eventually develop.

2-6. The streptococci are such a heterogenous group that neither morphological, physiological, nor immunological studies have been completely successful in classifying all species. Alpha or beta hemolysis exhibited on blood agar affords the best tentative separation of *Streptococcus* species and an indication of pathogenicity. Streptococci which produce a beta hemolysin show a clear, colorless, well-defined zone of hemolysis around the colony. Alpha hemolysin produces a greenish discoloration around the colony. The zone of hemolysis may have irregular or ill-defined margins because the red blood cells within the zone are only partially lysed.

2-7. *Streptococcus* colonies that produce alpha hemolysis must be carefully differentiated from those of *Diplococcus pneumoniae*, which also produce a "greening" of the medium by alpha hemolysis. The technique of differentiating between the two is covered in our discussion of diplococci in the next section. Some streptococci form a kind of hemolysin, termed alpha prime, in which the zone of hemolysis appears hazy and poorly defined. It is neither a clearcut alpha nor beta hemolysin. It is identifiable by an extension or enlargement of the zone of hemolysis around the colony when the blood agar culture is refrigerated for a 24-hour period. So-called "gamma" streptococci produce a hemolysin under certain experimental conditions, but no zone of hemolysis appears on conventional blood agar plates in the laboratory. We, therefore, record these gamma strains as nonhemolytic, as indicated in foldout 1. (Foldouts 1–8 are located in the separate inclosure to this volume.)

2-8. Along with the several types of hemolysin, streptococci produce a fibrinolytic enzyme, streptokinase, which is capable of breaking down fibrin clots. This may play a role in overcoming body defenses. Unlike the staphylococci, the streptococcal forms are catalase-negative—one major feature that distinguishes the genera (foldout 1). Beta hemolytic streptococci have long been known for their erythrogenic toxin associated with the skin rash of scarlet fever, a disease of children prevalent before the discovery of antibiotics.

2-9. **Classification of the Streptococci.** Several schemes for classifying the streptococci have been proposed. Two of the more widely used schemes are those devised by Lancefield and Sherman. The Lancefield classification is based on the antigenic structure of the organisms. The composition of a carbohydrate antigen—"C" substance—is different for each immunologic group in this series. The Sherman system has as its basis a compilation of common physiological properties, the most important of which is the temperature limit for growth.

2-10. The Lancefield serological groups are designated by the letters A through O. Groups A and C contain most of the human pathogens. These and nine other serological groups produce hemolysis; accordingly, they are referred to as the "hemolytic" streptococci. Organisms in Group D, including the enterococci, may be either hemolytic or nonhemolytic. Group N organisms are nonhemolytic. Currently about 19 species of *Streptococcus* are recognized. In the Sherman

TABLE 1
GROUP DIFFERENTIATION OF *STREPTOCOCCUS* SPECIES

Group	*Hemolysis*	*Growth at*		*Growth in*
		10°C.	45°C.	6.5% NaCl
Pyogenes (Gp A and C)	Beta or alpha	-	-(1)	-
Viridans (4)	Alpha or none	-(2)	+(3)	-
Enterococci (Gp D)	Alpha, beta or none	+	+	+
Lactic (Gp N)	None	+	-	-

(1) Exceptions with *Streptococcus sanguis.*
(2) Exceptions with *Streptococcus uberis.*
(3) Exceptions with *Streptococcus mitis.*
(4) Members of the Viridans group belong to Lancefield groups A, D, G, and K. Some strains cannot be grouped serologically.

system these species are divided into four major groups:

- Pyogenes.
- Viridans.
- Enterococcus.
- Lactic.

In table 1 the distinguishing cultural characteristics are portrayed for streptococci of the Sherman classification. The corresponding Lancefield groups are shown parenthetically.

2-11. The most important member of the *pyogenes* group clinically is *Streptococcus pyogenes* (Lancefield Group A). This species produces beta-hemolysis on blood agar. The organism grows at the usual cultural temperatures, but not at 10° C. or 45° C. The *viridans* group does not give beta-hemolysis on blood agar. As a rule, no growth is obtained at 10° C. Many species produce an alpha-hemolysis, but the effect on red blood cells depends upon the species origin of the red blood cells used, pH, and temperature. Viridans streptococci are found most frequently as normal flora in the human respiratory tract.

2-12. The *enterococcus* group yields variable hemolysis on blood agar. Growth generally takes place at both 10° C. and 45° C. Although these organisms are considered normal flora of man's intestinal tract, they can be pathogenic when introduced into the bloodstream, meninges, urinary tract, and various other tissues. Enterococci differ from the remainder of the streptococci in that they multiply in broth media containing 6.5 percent sodium chloride. This group is also quite resistant to most antimicrobial agents. The *lactic* group is characterized by variable hemolytic reactions on blood agar and growth at 10° C. but not 45° C. The lactic organisms are nonpathogenic. They are present in milk, and are often responsible for its souring.

2-13. Members of the genus *Peptostreptococcus*, which we noted earlier have been recovered from infections in various organs of the body, are generally considered apart from the streptococci of the two classifications discussed above. Of the 13 known species, only 3 are of medical interest: *P. anaerobius, P. foetidus,* and *P. putridous.* These organisms are catalase-negative like members of the genus *Streptococcus,* and they also produce a variety of hemolysins. A fetid odor and gas production on high protein media, along with the requirement for anaerobiosis, are distinguishing characteristics of the group.

2-14. **Laboratory Identification.** Beta hemolytic *Streptococcus* spp. are usually identified on the basis of cultural and physiological characteristics. Table 2 lists pertinent information on those groups responsible for the majority of human streptococcal infections. Note that the reactions obtained in *Streptococcus faecalis* (SF) broth and sodium hippurate broth do not separate Group A from Group C (human) streptococci. However, this degree of differentiation is not usually necessary since infections by members of both groups generally respond to identical antimicrobial agents.

2-15. The Bacitracin screening technique (foldout 1) is often useful in distinguishing between Lancefield's Group A and other groups of

TABLE 2

CHARACTERISTICS OF BETA HEMOLYTIC STREPTOCOCCI RESPONSIBLE FOR HUMAN INFECTION

Lancefield	*Type Species (Bergy-1957)*	*Source*	*Disease*	*Surface Colonies Blood Agar*	*Subsurface Colonies Blood Agar*	*SF Medium*	*Sodium Hippurate*
Group A	*S. pyogenes*	Human infections and carriers (usually respiratory).	Erysipelas, impetigo, septic sore throat, scarlet fever, rheumatic fever, acute glomerulonephritis, puerperal sepsis, septicemia, etc.	Grayish-white, opaque to translucent; hard, with tendency for whole colony to move on probing; hemolytic zones approximately 2 mm.	Lens or disc-shaped with 2 mm. zone of hemolysis.	No growth	Negative
Group B	*S. agalactiae*	Bovine infections and milk. Human intestinal, genitourinary, and respiratory tracts.	Urinary tract infections, peritonitis, endometritis, wound infections, rarely septicemia.	Gray, translucent, with soft texture; slight hemolytic zone.	0.5 mm. zones of hemolysis after 24 hours; 0.1 mm. zone after 48 hours. Double ring hemolysis following refrigeration overnight.	No growth	Positive
Group C (human)	*S. equisimilis*	Upper respiratory and intestinal tracts of humans.	Suppurative lesions of man, usually in mixed infections. Throat infections.	Indistinguishable from Group A.	Indistinguishable from Group A.	No growth	Negative
Group D (enterococci).	*S. faecalis var. zymogens* *S. durans*	Intestinal and genitourinary tracts of humans. Milk and milk products.	Same as Group B.	Gray, somewhat translucent, soft; hemolytic zones slightly wider than the colonies.	Hemolytic zones approximately 3 to 4 mm.	Growth, with acid reaction	Negative

NOTE: *Streptococcus faecalis* and *S. liquefaciens* are also members of Serologic Group D; however, these species usually produce alpha hemolysis on blood agar. Also *S. faecalis* is occasionally observed to be nonhemolytic (gamma). These forms represent the only nonbeta species that may be uniformly grouped serologically.

the beta hemolytic streptococci. We first streak a blood agar plate with a pure culture of the isolated organism; then we place a one- or two-unit bacitracin-impregnated paper disc on the surface. Susceptibility or resistance to the antibiotic is read after incubation of the culture for 18 to 24 hours in a candle jar at 37° C. Of those beta hemolytic isolates showing antibiotic susceptibility, about 85 percent will be Lancefield's Group A. Almost 100 percent of the resistant forms will be *other* than Group A.

2-16. We see in table 2 that SF broth is selective for *Streptococcus faecalis* and other members of Group D. Sodium azide in the medium prevents the growth of other streptococcal groups. After incubation for 24 hours at 37° C. the presence of growth, coupled with an indicator change from purple to a yellowish-brown, indicates a positive reaction. Similarly, sodium hippurate broth distinguishes Group B streptococci from groups A, C, and D. The inoculated medium is incubated for 48 hours and then tested for the presence of benzoic acid. This compound is formed by the hydrolysis of sodium hippurate. The formation of a stable precipitate after we add 12 percent ferric chloride indicates a positive reaction.

2-17. Foldout 1 may be consulted for confirmation of the well-recognized species. From a practical standpoint, it is necessary only to differentiate *Streptococcus faecalis* from other alpha and gamma (nonhemolytic) streptococci. *Streptococcus faecalis* is resistant to penicillin, streptomycin, and sulfonamides, while the other alpha and gamma strains are sensitive to these agents. Table 3 presents additional data of help in identifying the various alpha and gamma *Streptococcus* species that cause disease in man.

3. Diplococcus Infections of Man

3-1. The genus *Diplococcus* has but one species that is pathogenic for man. It is *Diplococcus pneumoniae*, or more commonly—the pneumococcus. The taxonomy of this organism is as follows:

Order IV. *Eubacteriales*
  Family X. *Lactobacillaceae*
    Tribe I. *Streptococceae*
      Genus I. *Diplococcus*
        Species. *D. pneumoniae*

3-2. **Clinical Significance.** Pneumococci are the cause of approximately 80 percent of the cases of lobar pneumonia and roughly 1.5 percent of the bronchial pneumonia in man. The sputum is usually bloody or rusty and has a thick viscous consistency. In the early stages of pneumococcal pneumonia, bacteremia may be present. Pneumococci can invade other tissues, either as complications of pneumonia or as independent and primary infections. From the respiratory tract the organisms frequently spread to the sinuses and middle ear. A meningitis may result from blood stream infection following pneumonia.

3-3. Virulent pneumococci are generally spread by asymptomatic carriers. The pneumococci are "opportunist pathogens" in that they apparently do not invade except when an individual's resistance to infection declines. Pneumococcal pneumonia can hasten the fatal termination of such diseases as influenza, tuberculosis, congestive heart failure, and cancer. The organism has also been implicated in postsurgery complications.

3-4. **General Characteristics.** The pneumococci are small, slightly elongated cocci arranged in pairs—diplococci. In young cultures the cells are frequently lancet-shaped, with the adjoining ends flattened or slightly curved. The organisms may occur singly, in pairs, or in short chains. Pneumococci are typically Gram-positive, but older cultures become Gram-negative and show a tendency to lyse spontaneously. They are nonmotile and nonsporeforming. Virulent pneumococcal cells are surrounded by a well-defined polysaccharide capsule that is prominent when the organisms are observed in tissue exudates.

3-5. The virulence is, in part, dependent upon this capsule which functions as a barrier against phagocytosis. Various strains possess antigenically distinct polysaccharides in the capsule, each inciting the formation of antibodies in the host. The antipolysaccharide antibodies are specific, because once formed they will react typically with only the particular type of capsular polysaccharide that initiated their production. Such reactions result in the destruction and removal of the capsule from the cell wall, thereby rendering the pneumococcus vulnerable to phagocytosis.

3-6. Gram-stained smears of sputum or blood specimens reveal the typical Gram-positive, lancet-shaped diplococci shown in figure 1. The organisms may also be seen in spinal fluid sediment, pleural fluids, or other exudates from infected tissue. The presence of a capsule is revealed in Gram-stained smears as a thick halo around the cell when observed under reduced light. The polysaccharide material is more easily seen by performing a capsule stain or an India ink preparation.

3-7. Pneumococcal strains require an enriched medium for growth. The standard blood agars and broth media such as thioglycollate, brain-heart infusion, Todd-Hewitt, and trypti-

TABLE 3

IDENTIFICATION OF ALPHA AND GAMMA *STREPTOCOCCUS* SPECIES

Species	*Source*	*Pathogenicity*	*SF Broth*	*Mannitol*	*Inulin*	*Reduction Methylene Blue Milk*	*Colonies on Blood Agar*	*Growth in 5% Sucrose Broth*	*Colonies 5% Sucrose Agar*
S. salivarius	Respiratory tract	Mild respiratory tract infections, subacute bacterial endocarditis, etc.	No growth	-	+	-	Alpha 24 to 48 hours	No change	Large, mucoid raised.
S. mitis	Respiratory tract	Mild respiratory tract infections, subacute bacterial endocarditis, etc.	No growth	-		-	Alpha 24 to 48 hours	No change	Small convex.
S. sanguis	Blood	Subacute bacterial endocarditis	No growth	-	+	-	Alpha 24 to 48 hours	Jelling of broth	Small convex.
Streptococcus MG	Respiratory tract	Secondary invader primary atypical pneumonia	No growth	-	-	-	No hemolysis 24 hrs. Alpha 24 to 48 hours	No change	Small, convex, fluorescent in ultraviolet light.
S. faecalis (enterococcus Group D)	Intestinal and genitourinary tracts	Urinary tract infections, peritonitis, endometritis, subacute bacterial endocarditis	Growth	+	-	+	No hemolysis 24 hrs. Slight alpha 48 hours	No change	Small convex.

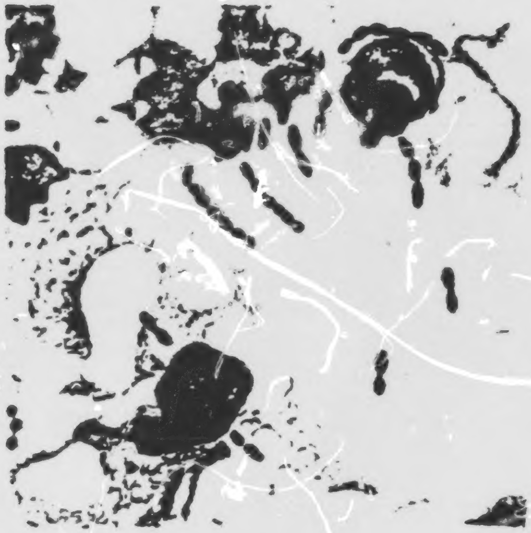


Figure 1. *Diplococcus pneumoniae*—Gram stain of blood culture smear.

case soy generally yield luxuriant characteristic growth. Pneumococci are aerobes, but they grow better under increased carbon dioxide pressure such as in a candle jar, particularly on primary isolation. On blood agar after 18 to 24 hours' incubation at 37° C., typical virulent strains of pneumococci form flat, smooth, transparent, slimy, or mucoid colonies that may be concave. Colonies measure 0.5 to 1.5 mm. in diameter, and are generally alpha hemolytic. The pneumococci produce an autolysin which tends to dissolve their own colonies after prolonged incubation.

3-8. There are three major cultural phases of the pneumococcus. There are the mucoid (M), the smooth (S), and the rough (R) phases. The mucoid (M) form represents normal colony appearance. It is due to the formation of a type-specific polysaccharide in the capsular material. The smooth (S) form has an even, shiny appearance. Its colonies lack the polysaccharide material of the mucoid phase. The rough (R) form has a rough or textured surface, and the colony is usually slightly smaller. Like the smooth form, it does not possess the mucoid type polysaccharide. Most pathogenic pneumococci are encapsulated and form the M colony. Loss of the capsule is accompanied by a loss of virulence and antigenic specificity.

3-9. **Laboratory Identification.** Isolated colonies suspected to be pneumococci should be evaluated by two techniques: (1) A sodium desoxycholate (or bile) solubility test to observe for lysis of the cells and (2) determination of optochin sensitivity. In the sodium desoxycholate solubility test, we add a few drops of 10 percent sodium desoxycholate to a growing, buffered, broth culture (pH 7.4). Lysis of the cells in the broth will produce a visible clearing within 10 minutes if the organisms are pneumococci. The optochin or Taxo P® sensitivity disks (ethylhydrocupreine hydrochloride) are placed on the heavily inoculated area of a blood agar plate. After 18 to 24 hours pneumococcal cultures exhibit a large clear zone of inhibition around the disk (no growth), as seen in figure 2.

3-10. Pneumococci may also be quickly and accurately identified by serological typing. If Gram stains of clinical material show the pneumococcus in relatively pure culture, samples of the specimen may be reacted with type-specific antisera on a glass slide and observed for the Neufeld-Quellung reaction. We expose the organism to type-specific antisera for the strains most commonly responsible for human infections. When a pneumococcus type is mixed with its specific antipolysaccharide antibody, the capsule appears to swell and becomes markedly defined under microscopic observation. This is the basis of the Neufeld-Quellung reaction, as shown in figure 3. Table 4 shows how we can distinguish between pneumococci and the morphologically similar alpha streptococci by employing a combination of tests—bile solubility, optochin sensitivity, and serological studies.

4. The Corynebacteria

4-1. The only true pathogenic species of *Corynebacterium* is *C. diphtheriae*. The three varieties of this species—*gravis*, *intermedius*, and *mitis*—cause the disease diphtheria. The

Figure 2. Culture of *Diplococcus pneumoniae* showing zone of inhibition.

1.07

Figure 3. Neufeld-Quellung reaction.

commonly encountered saprophytic Gram-positive bacilli that resemble *C. diphtheriae* morphologically are referred to as "diphtheroids." They normally inhabit the mucous membranes of the respiratory tract and conjunctiva. Typical of the diphtheroids are *Corynebacterium diphtheriticum* (also called *Corynebacterium hoffmanii*), *Corynebacterium striatum*, and *Corynebacterium xerosis*. These members of the genus are not associated with diseases of man. Corynebacteria are classified taxonomically as follows:

Order IV. *Eubacteriales*
  Family XII. *Corynebacteriaceae*
    Genus I. *Corynebacterium*

4-2. **Clinical Significance.** *Corynebacterium diphtheriae* is found in the upper respiratory tract of infected individuals and asymptomatic carriers. The organism is isolated rarely from the skin or wounds. The bacilli are spread by nasal or oral droplets, or by direct contact. The virulent bacilli invade the mucous membranes, multiply rapidly, and produce a powerful exotoxin. Absorption of the toxin by the mucous membrane yields an acute inflammatory response and destruction of the epithelium. The accumulation of fibrin, red blood cells, and white blood cells results in the formation of a gray, clotted film or "pseudomembrane" that covers the tonsils, pharynx, or larynx.

4-3. As the disease progresses, the toxin is absorbed by other tissues. The organisms remain localized in the upper respiratory tract. In clinically typical diphtheria, lesions and pseudomembranes in the throat usually yield large numbers of the characteristic bacilli upon direct microscopic examination of smears. It is the exotoxin, disseminated to the blood and deeper tissues, which accounts for the symptoms of systematic involvement.

4-4. **General Characteristics.** The corynebacteria are slender, Gram-positive rods measuring from 1 to 6 microns in length and 0.3 to 0.8 micron in width. They usually exhibit considerable pleomorphism. In addition to straight or slightly curved rods, club or dumbbell-shaped forms are common. The diversity of shapes stems from the irregular distribution of cytoplasmic granules—metachromatic or Babes-Ernst granules—that distort the cell wall.

4-5. In smears stained with Loeffler's alkaline methylene blue and similar dyes, the metachromatic granules appear as deeply stained bodies against a lighter cytoplasm. This contrast gives the cell a banded, barred, or beaded appearance. Corynebacteria are characteristically arranged in palisades, but V- or Y-shaped branching forms may also occur. Microscopic groupings have been compared to "Chinese letters" or "piles of

TABLE 4
DIFFERENTIATION OF PNEUMOCOCCI FROM ALPHA STREPTOCOCCI

Organism	*Hemolysis*	*Colonial Morphology*	*Capsules*	*Quellung Reaction*	*Optochin (Taxo-P)®*
Pneumococci	Alpha	Small, flat, shiny or mucoid, depressed center and concentric rings	Usually present	Positive	Sensitive
Alpha Streptococci	Alpha	Small, raised dome-shaped, smooth, translucent, or opaque	Absent	Negative	Resistant

NOTE: Colonies of pneumococcus type III are generally larger, raised, and more mucoid, with a tendency to form confluent growth. These forms resemble droplets of oil.

203

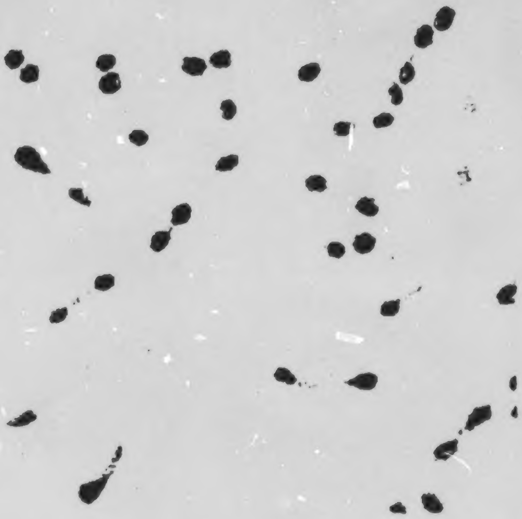


Figure 4. *Corynebacterium diphtheriae.*

matches." as shown in figure 4. It is important to remember that saprophytic diphtheroids may resemble *Corynebacterium diphtheriae;* however, diphtheroids are usually short, thick, uniformly stained rods. In most cases, the diphtheroids exhibit little or no pleomorphism, as we can observe in figure 5.

4-6. Good growth of the corynebacteria is usually obtained on enriched media such as blood agar, but Loeffler's serum slants and potassium tellurite agar plates are recommended for primary isolation of diphtheria bacilli. Corynebacteria are aerobic, and they develop well at 37° C. Typical growth is formed within 18 to 24 hours on Loeffler's agar. On potassium tellurite medium, characteristic growth of colonies usually requires 24 to 48 hours' incubation.

4-7. Typical colonies of *Corynebacterium diphtheriae* are described in table 5, which was extracted from AFM 160–52. The organism may also develop smooth (S), rough (R), intermediate (Sr, SR, sR), and dwarf (D) forms. The smooth form is not generally present on potassium tellurite agar. As the name implies, the intermediates produce gradations in colony texture between the very smooth (S) and extremely rough (R) forms. The dwarf (D) colonies are minute, measuring about 0.2 mm. in diameter. It is possible to find colonies of smooth, rough, intermediate, and dwarf morphology in routine laboratory investigations, because all have been isolated from cases of diphtheria.

4-8. Laboratory Identification. In searching for pathogens on blood agar, Loeffler's, and tellurite agar, we use comparative colony descriptions supplemented by microscopic study of slides stained with Loeffler's alkaline methylene blue or the Gram stain. *Corynebacterium diphtheriae* usually grows more rapidly and more luxuriantly on Loeffler's serum medium than do contaminating microorganisms of the respiratory tract, with the exception of the diphtheroids. The microscopic morphology of diphtheria bacilli is usually characteristic on Loeffler's medium, yet often atypical on tellurite agar. We use the latter medium because it is inhibitory to many Gram-positive cocci, and particularly to Gram-negative organisms. Diphtheroids and staphylococci will grow on it, however.

4-9. *Corynebacterium diphtheriae* can only be identified with certainty by demonstrating the production of toxin. Observation of typical colonial and microscopic morphology is merely presumptive. Carbohydrate fermentation studies are unreliable. The toxigenicity of a given strain may be determined by either in-vivo or in-vitro testing, but since the former requires the use of laboratory animals, the in-vitro procedure is simpler and more practical.

4-10. In-vitro virulence testing involves a specific antigen-antibody reaction. A piece of filter paper (1 x 7 cm.) that has been dipped in diphtheria antitoxin of a potency equal to 500 units/ml. is placed on Klebs-Loeffler (KL) virulence medium while the agar is still soft. We press the paper into the medium, allow the agar to harden, and streak the surface of the medium with material from the suspected colony along a single line at a right angle to the filter paper. If the *Corynebacterium* species is toxigenic, white lines of a precipitate will be observed in the agar

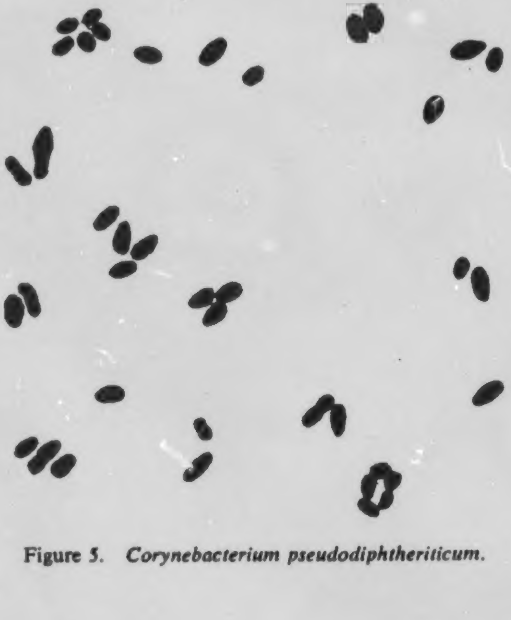


Figure 5. *Corynebacterium pseudodiphtheriticum.*

TABLE 5
COLONY CHARACTERISTICS OF CORYNEBACTERIA

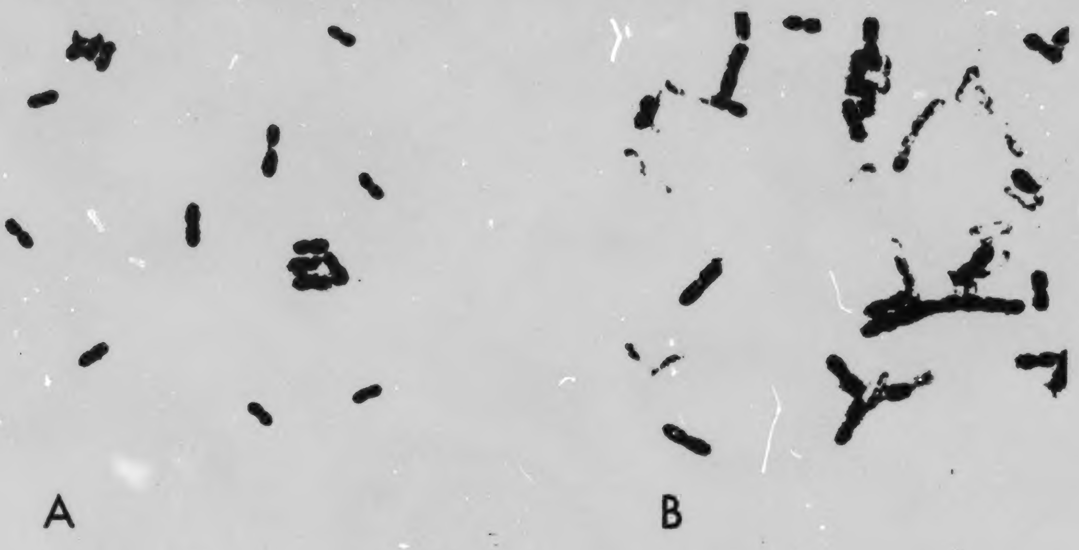
Species	*Blood Agar (18 to 24 hours)*	*Loeffler's Serum Medium (18 to 24 hours)*	*Potassium Tellurite Agar (48 hours)*
C. diphtheriae, var. *gravis*	Small, gray, dull opaque colonies which are nonhemolytic.	Circular, convex, cream-colored colonies with raised centers.	Flat, irregular, slate-gray colonies with a dull surface (2 to 3 mm. in diameter).
C. diphtheriae, var. *intermedius*	Small, gray, dull opaque colonies which are nonhemolytic.	Similar to var. *gravis*.	Circular, convex colonies with brownish-gray color against a white background (0.2 to 0.3 mm. in diameter).
C. diphtheriae, var. *mitis*	Small, gray, dull opaque colonies which are usually hemolytic.	Similar to var. *gravis*.	Black, convex colonies with a glistening surface (1.0 to 1.5 mm. in diameter).
Diphtheroid bacilli	*Colonies considerably variable and nonhemolytic.	Similar to var. *gravis*.	Flat colonies, light gray or dark in center with white or gray translucent periphery.

* Certain diphtheroids form colonies that are dull and flat with a slightly rough surface Others form staphylococcus-like colonies that are large, grayish white and smooth surfaced. Yellowish pigmented forms resembling *Neisseria spp.* are occasionally encountered.

after a period of incubation. The precipitate appears at the boundary between the diffusing antibody (antitoxin) and the toxin (antigen) liberated by the culture. The reaction on the plated medium is easily observed over a dark background under oblique light. Nontoxic strains will not exhibit lines of precipitation, because no toxin is produced to react with the antitoxin.

TABLE 6
CHARACTERISTICS OF DIPHTHEROIDS

Name	*Blood Agar (18 to 24 hours)*	*Loeffler's Serum (18 to 24 hours)*	*Loeffler's Methylene Blue Stain*
C. enzymicum	Small, colorless, moist, or yellow-white	Fine, moist, confluent growth	Similar to *C. diphtheriae*
C. striatum	Circular, white, smooth, entire. Fifth day, slight hemolysis	Slightly raised, convex	Coccoid and long filamentous forms. Large metachromatic granules appear as regular bars
C. xerosis	Minute, circular, almost transparent, raised, smooth, pearly white	Thin, gray, adherent	Exhibits polar staining
C. pseudodiphtheriticum	Gray to cream, smooth, entire	Gray to cream, smooth entire	Fairly uniform in size. No swollen ends; not barred. Granules usually absent
C. acnes	Very small, circular, transparent, rose colored (late)	Small, gray, (late rose colored)	Quite long, club shaped; sometimes possess rudimentary branches



A. YOUNG CULTURE
B. AFTER 2 DAYS' INCUBATION AT 37°C

Figure 6. *Listeria monocytogenes.*

4-11. It is seldom necessary to distinguish between species of the diphtheroids. The task of differentiating diphtheroids from the diphtheria bacillus is made easier, however, if you are familiar with the colonial and cellular morphology described in table 6. The anerobic diphtheroids are rarely found in disease processes, but they have been implicated in cases of subacute bacterial endocarditis from time to time. *Corynebacterium acnes*, the most common anaerobic diphtheroid, has been confused with *Actinomyces* species. Since the actinomyces are catalase negative and the corynebacteria are positive, differentiation is not difficult.

5. Listeria Infections

5-1. *Listeria monocytogenes* is a little known relative of the corynebacteria isolated from rabbits in 1926. Morphological similarity to the diphtheroid forms is clearly shown in figure 6. Since the organism has been recovered in the laboratory on relatively few occasions, its prevalence as a disease agent in humans has not been fully established.

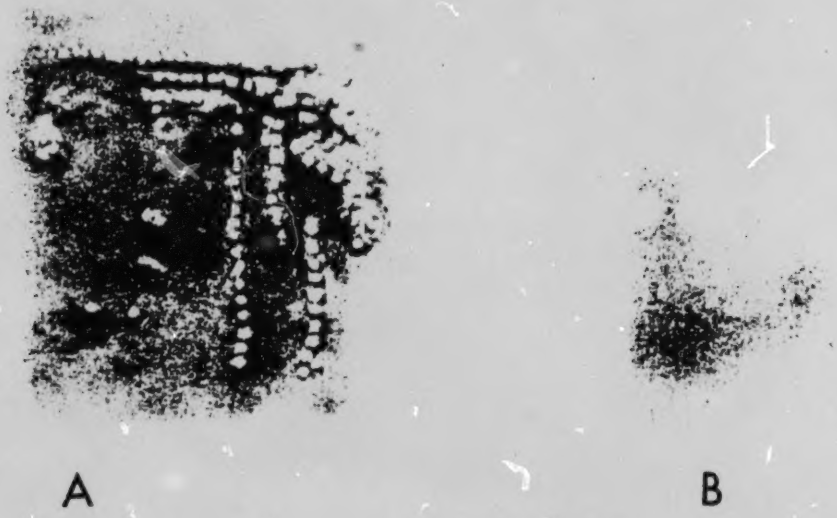
5-2. The disease listerosis, or "glandular fever," is an acute, benign lymphoblastosis of children and young adults. The microorganism has been implicated in cases of meningitis, septicemia, and abortion. Its taxonomic position is shown below:

Order IV. *Eubacteriales*
  Family XII. *Corynebacteriaceae*
    Genus II. *Listeria*

5-3. **General Characteristics.** *L. monocytogenes* is a Gram-positive, nonsporeforming rod with rounded ends. No capsule is formed. The cell measures 0.5 to 0.6 micron wide and 0.5 to 2.0 microns in length. The bacilli often form "palisades" on Gram-stained smears, as shown in figure 6A. Metachromatic granules are not observed. The organism is actively motile by means of peritrichous flagella.

5-4. Optimum incubation temperature is 37° C. This facultative anaerobe grows well aerobically in a neutral or slightly alkaline medium, as shown in figure 6B. It tolerates a high-concentration salt medium. It usually produces a beta hemolysis, but this characteristic is not constant. On sheep, human, or rabbit blood the colonies are small (0.8 to 2.0 mm. in diameter) and gray or white in color, as shown in figure 7. Hemolysis may not develop for 48 hours on human or rabbit blood, but on sheep blood it is detectable within 24 hours. On potassium tellurite medium the colonies are black, circular, and smooth; they measure about 0.25 to 0.5 mm. in diameter. Both rough and smooth colony forms can develop on the more common media.

5-5. **Laboratory Identification.** Isolation of the organism from blood, spinal fluid, and meconium can be made on a medium containing phenyleethanol agar, lithium chloride, and glycine. After incubation for 24 hours at 37° C. the plates should be examined with a hand magnifying lens. The colonies appear blue green in color. The motility of listeria can best be demonstrated by using a dextrose semisolid agar stab culture. Two



A. BY REFLECTED LIGHT
B. BY TRANSMITTED LIGHT

Figure 7. *Listeria monocytogenes.*

tubes should be inoculated; one to be incubated at 37° C. and the other at 25° C. Motility in the agar is more pronounced at 25° C. A ring forms just beneath the surface of the agar; cloudlike growth then extends downward from the ring.

5-6. Care must be taken in examining blood agar plates to insure that colonies giving beta hemolysis are not confused with beta hemolytic streptococci. A stained slide will show the difference in morphology. Listeria grows on tellurite agar and must not be mistakenly discarded as a diphtheroid. Foldout 2 shows that determination of catalase activity, a motility test, and scanning a slide for metachromatic granules will distinguish between *Listeria monocytogenes* and the corynebacteria diphtheroids.

6. Erysipelothrix Infections

6-1. *Erysipelothrix insidiosa* is the causative agent of the disease, erysipeloidosis, and has a predilection for the skin, joints, and endocardium. The disease is primarily an occupational one, affecting individuals who handle fish, shellfish, poultry, and certain meat products, particularly pork. The taxonomic classification of *Erysipelothrix* is as follows:

Order IV. *Eubacteriales*
  Family XII. *Corynebacteriaceae*
    Genus III. *Erysipelothrix*

6-2. **General Characteristics.** There is a morphological similarity between *Erysipelothrix insidiosa* and *Listeria monocytogenes.* The smooth erysipelothrix colonies reveal slender Gram-positive rods, measuring about 0.2 to 0.4 micron by 0.4 to 2.5 microns. The rods are straight or curved, with rounded ends. The cells from rough colonies may also appear as long filamentous structures.

6-3. *Erysipelothrix insidiosa* grows poorly on simple media. The addition of serum or glucose enhances growth. The rough colony form of growth is favored at 37° C., while the smooth colony is produced at 33° C. After a 24-hour incubation period, the small, rough colony has a matte-surface with an edge similar to anthrax colonies. The smooth colonies are round, water clear, and glistening. Many of these colonies produce an alpha hemolysis on blood agar after prolonged incubation. This genus is facultatively anerobic, although it tends to grow microaerophilically. It is catalase-negative.

6-4. **Laboratory Identification.** The provisional diagnosis of erysipeloidosis is based primarily on clinical findings, but the final diagnosis rests on the isolation and identification of *Erysipelothrix insidiosa* from suspected lesions. Biopsies of the lesion should be taken for culture, because positive cultures are rarely obtained from swab specimens. Initial cultivation should be started in a glucose broth, with subsequent subculture to blood agar plates. In a gelatin stab, the growth is filiform at first; then lateral radiating growth appears, lending a "test tube brush" appearance. On gelatin slants, the smooth (S) forms remain along the line of inoculation, whereas the rough (R) form spreads out over the surface.

6-5. Differentiation of the three genera *Cory-*

nebacterium, Listeria, and *Erysipelothrix* is important. Foldout 2 lists some of the characteristics which help in distinguishing them. The most clear-cut difference is the absence of catalase production in *Erysipelothrix*, but motility and microscopic morphology are helpful.

7. Bacillus Anthracis

7-1. Among the members of the genus *Bacillus*, only *Bacillus anthracis* (anthrax bacillus) is clearly established as a pathogen. The saprophytes, *B. subtilis, B. megaterium,* and *B. cereus* are important, however, because their microscopic and colonial morphology is often indistinguishable from that of the anthrax bacillus. The saprophytic species from soil and air frequently occur as laboratory contaminants. The taxonomic classification of the bacilli is as follows:

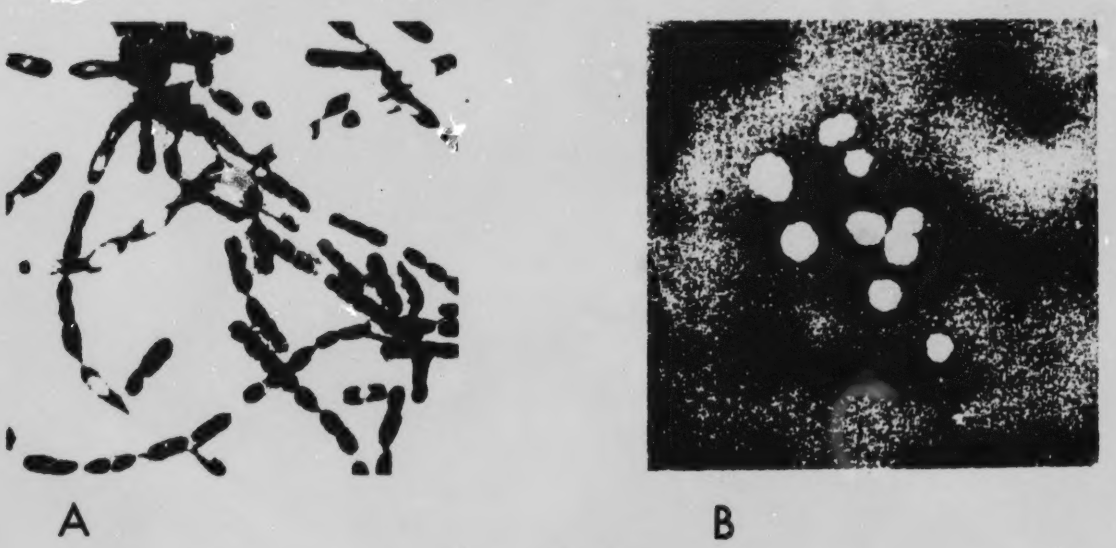
Order IV. *Eubacteriales*
  Family XIII. *Bacillaceae*
    Genus I. *Bacillus*

7-2. **Clinical Significance.** Anthrax is primarily a disease of herbivorous animals incurred during grazing on pastures contaminated by anthrax spores. Viable spores germinate in the intestinal tract or the buccal mucosa, and the bacilli are disseminated via the lymphatics to the bloodstream and deeper tissues. Infections in man are almost always of animal origin, occurring in butchers, herdsmen, woolhandlers, tanners, and other occupational groups dealing with infected animals or their products.

7-3. The organisms may enter through the skin, the respiratory tract, or the intestinal mucosa. Cutaneous anthrax, the most common form of human infection, results from direct contact with infected tissue, hides, hairs, or bristles. Skin lesions may liberate bacilli, resulting in septicemia and widespread involvement of internal organs. Primary pulmonary anthrax arises from inhalation of spores disseminated into the air during the processing of infected materials, especially animal hides and fleece. Symptoms of pneumonia often progress to a fatal septicemia in untreated cases. Intestinal anthrax results from consumption of insufficiently cooked meat from infected animals or from ingestion of foods contaminated with spores. Pulmonary anthrax and infections of the intestinal tract are very rare in man, but the latter are the most common form of the disease in animals.

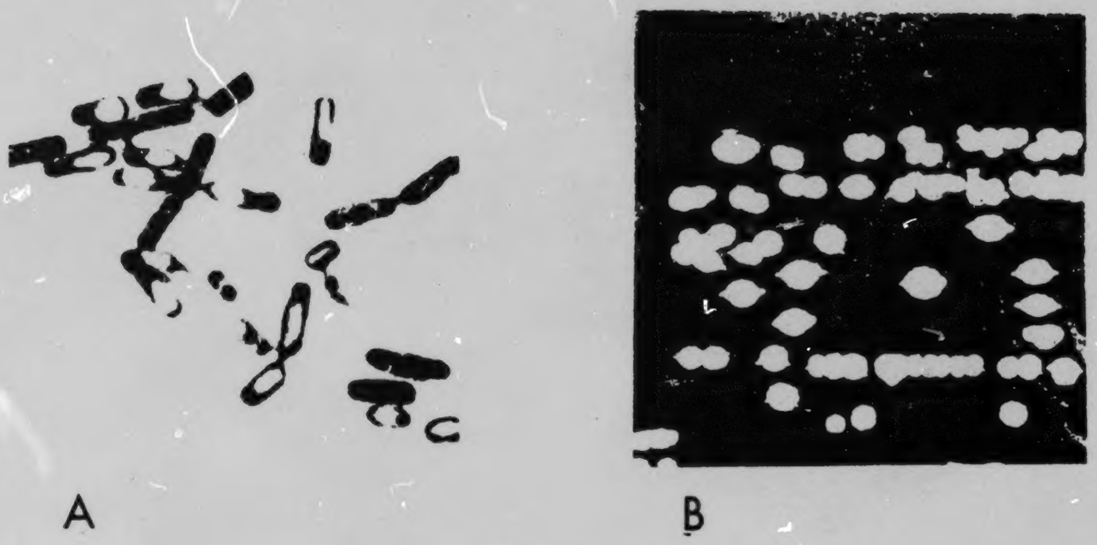
7-4. *Bacillus anthracis* produces no soluble exotoxin or endotoxin. Virulence is apparently associated with the ability to form a capsule. The capsule is composed of polypeptide made up of d-glutamic acid units instead of the polysaccharides common to capsules of most other bacteria.

7-5. The vegetative cells of *Bacillus* spp. are no more resistant to disinfectants or heat than other bacteria, but the spores are highly resistant. Anthrax spores have been known to survive for decades in soil. The spores ordinarily survive boiling for several minutes and exposure to most disinfectants must be prolonged to be effective. Standard sterilization temperatures and the usual periods of heating in the autoclave successfully destroy all bacillus spores.



A. MORPHOLOGY
B. COLONY

Figure 8. *Bacillus anthracis.*



A. MORPHOLOGY
B. COLONY

Figure 9. *Bacillus subtilis.*

7-6. **General Characteristics.** Members of the genus *Bacillus* are large, Gram-positive, spore-forming rods, usually occurring in chains, as shown in figure 8A. Individual cells range between 1 to 1.25 microns in width and 3 to 10 microns in length. Bacillus anthracis is encapsulated and nonmotile, but the many saprophytic forms lack capsules and are usually actively motile. The encapsulated cells of *B. anthracis* found in direct smears of clinical specimens are rarely observed in smears from laboratory cultures. Most bacilli appear as long, straight-sided rods with curved ends, but the cells of *B. anthracis* often possess swollen, square, or concave ends. *B. anthracis* does not sporulate in living tissue, but spores are formed when the organism is exposed to the external environment.

7-7. In Gram stained smears of exudates and blood, *B. anthracis* is seen as large Gram-positive bacilli, usually in chains of two to six cells. The bacilli may be numerous in blood smears from generalized anthrax. The capsules cannot be clearly observed in Gram-stained preparations, yet their presence may be noted as imperfectly stained, granular halos with ragged edges. In films of exudates, blood, and tissue impressions stained with Wright's or Giemsa's stain, anthrax bacilli appear bluish-black, with a clearly defined, pinkish capsular substance surrounding them.

TABLE 7
DIFFERENTIATION OF BACILLUS ANTHRACIS FROM SAPROPHYTIC (PSEUDOANTHRAX) BACILLUS SPECIES

Organism	*Colonies on Blood Agar*	*Hemolysis*	*Motility*	*Capsule*	*Mouse or Guinea Pig Virulence*
Virulent *B. anthracis*	Rough, dull, and irregular ("frosted glass")	-	-	+	+
Avirulent *B. anthracis*	Rough, dull, and irregular ("frosted glass") or smooth or mucoid	-	-	-	-
Saprophytic (pseudoanthrax) *Bacillus spp.*	Rough, dull, flat, irregular, and often spreading. Occasionally smooth, shiny, and compact	+ (usually)	+ (usually)	-	-

7-8. All *Bacillus* spp. propagate rapidly as aerobes on simple basic media, and the addition of special enrichments such as blood or carbohydrates does not substantially improve growth. Growth occurs over a wide range of temperature for most species, especially the saprophytic forms. The optimum incubation temperature for *B. anthracis* is 37° C. Spores are formed abundantly at 32° C. to 35° C.

7-9. On blood agar after 18 to 24 hours' incubation, typical colonies of the anthrax bacillus are 2 to 3 mm. in diameter and appear gray-white, opaque, and dull, with irregular edges and a rough "frosted glass" appearance (fig. 8B). Since *B. subtilis, B. cereus, B. megaterium,* and other saprophytic species may exhibit the same colonial morphology, they are often referred to as pseudoanthrax bacilli or "anthroids." Morphology of *B. subtilis* is seen in figure 9A.

7-10. **Laboratory Identification.** Hemolysis is an important basis for differentiation. Anthrax colonies are nonhemolytic or weakly hemolytic on blood agar (fig. 8B), while pseudoanthrax forms are usually surrounded by a definite zone of hemolysis, as shown in figure 9B. Table 7 sums up the morphological differences that provide the basis for laboratory identification. The capsule is a key factor only in smears taken directly from tissue or body fluids. Motility testing is useful, although this property is variable to some extent among the saprophytic species.

7-11. Animal virulence tests confirm the pathogenicity of organisms isolated in the laboratory. Fresh specimens of blood, sputum, macerated tissue, exudates from lesions, or saline suspensions of 24-hour nutrient agar slant cultures are inoculated subcutaneously into the abdomen of white mice or guinea pigs. Large inocula are not necessary because less than 100 organisms will usually prove lethal. After 36 hours, animals give evidence of illness and exhibit doughy swellings around the area of inoculation. Death may occur in less than 48 hours, but 3 to 4 days are ordinarily required.

7-12. In laboratory animals dying from anthrax, a yellow gelatinous exudate will be found in the subdermal region near the point of injection. Often this exudate extends over large areas of the abdominal subcutaneous connective tissue. The blood vessels and the mesenteries show marked congestion and hemorrhages near the injection site. The spleen is greatly enlarged and much darker than normal. Although anthrax bacilli can usually be demonstrated in stained smears of blood or any of the tissues, examination of the spleen is most productive.

8. The Clostridia

8-1. *Bergey's Manual of Determinative Bacteriology* (7th Ed) lists 93 different species of *Clostridium*. Some of these species are further subdivided into serological types. Fortunately, not all are encountered in routine bacteriological examination, but several species are of great medical importance. Unlike the other members of the family, *Bacillaceae*, the genus *Clostridium* consists of obligate anaerobes. Taxonomic classification is as follows:

Order IV. *Eubacteriales*
  Family XIII. *Bacillaceae*
    Genus II. *Clostridium*

8-2. **Clinical Significance.** Clostridia are distributed worldwide in soil and the intestinal tract of animals. The pathogenic species are responsible for botulism, tetanus, and gas gangrene. The first two diseases are intoxications caused by exotoxins whose potency matches or exceeds that of snake venom. Gangrene is a true infection accompanied by severe and extensive tissue destruction.

8-3. *Clostridium tetani* is the etiologic agent of tetanus (lock jaw). This disease follows the introduction of tetanus spores (from soil or feces) into puncture wounds, burns, surgical sutures, or traumatic injuries. If anaerobic conditions prevail in the wound, the spores germinate to form the vegetative bacilli which produce the toxin whose action precipitates the disease. The rapidly absorbed toxin acts on the tissue of the spinal cord and peripheral motor nerve endings. The toxemia is evidenced by muscle spasms near the site of infection and subsequent spasms of the jaw muscles (lockjaw). The nerves of other voluntary muscles are progressively involved, causing tonic spasms and convulsions.

8-4. *Clostridium botulinum* is responsible for an often fatal type of food poisoning called botulism. Outbreaks occur following the ingestion of food in which the organism has produced a highly potent exotoxin. In the anaerobic environment of a foodstuff, the spores germinate to form vegetative cells which, in turn, produce the toxin. The spores will withstand a temperature of 100° C. for at least 3 to 5 hours.

8-5. Toxin-containing foods may appear spoiled and rancid, and cans may be swollen due to gas formation by the organism. In some cases, however, the food appears entirely innocuous. The toxin itself is destroyed by heating the food at 100° C. for 10 minutes. Outbreaks of botulism are rare in the United States because of rigid quality control in commercial canning and food preservation. Nevertheless, cases of botu-

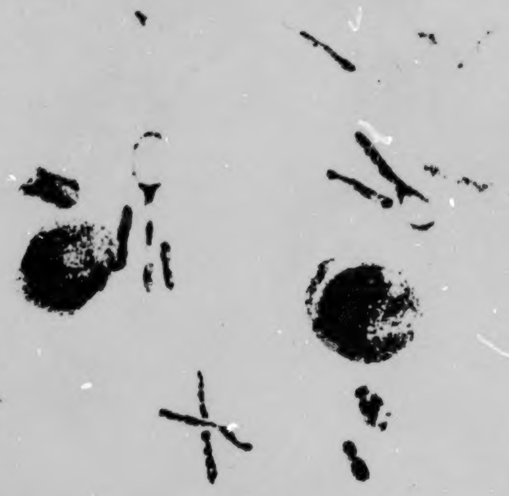


Figure 10. *Clostridium tetani.*

lism still occur from consumption of home-preserved foods containing *Cl. botulinum* exotoxin spores.

8-6. There are six antigenically distinct types of *Cl. botulinum* toxin designated A through F. Humans are affected by types A and E, and occasionally by type B. There is to date only one incident of poisoning in man by types C, D, and F. Type A toxin is the most poisonous substance known. From 18 to 48 hours following the consumption of toxic food, neurotoxic symptoms are evidenced by visual disturbances, inability to swallow, and speech difficulty. Progressive signs of bulbar paralysis are exhibited, and the disease can terminate fatally from respiratory failure and cardiac arrest.

8-7. The organisms most commonly causing gas gangrene are *Clostridium perfringens, Cl. novyi,* and *Cl. septicum. Cl. perfringens* has five toxin types designated as A through E. Type A produces the classic gas gangrene in humans. *Cl. novyi* is divided into four toxigenic types: A, B, C, and D. Type A, again, is implicated in gas gangrene of humans. *Cl. perfringens* is the most frequent cause of the disease, either singly or in combination with other anaerobes.

8-8. Gangrene develops as a complication of severe traumatic injuries, especially lacerated wounds accompanied by a compound fracture. In these injuries the blood circulation to a local tissue area is often impaired or destroyed. The resulting necrotic tissue, void of oxygen and rich in microbial nutrients, affords an ideal anaerobic environment in which clostridial spores germinate and multiply. The organisms actively metabolize tissue carbohydrates to acid and gas. The gangrenous process extends to other tissues primarily as a result of exotoxins excreted by the pathogenic clostridia. In addition, bacterial enzymes may exhibit hemolytic, necrotizing, and lethal effects on tissue cells.

8-9. Gas gangrene is usually a mixed infection of toxigenic (toxin-producing) and proteolytic (protein-destroying) clostridia and other Gram-positive or Gram-negative anaerobic organisms. The accessory organisms may contribute to the severity of infection. The antitoxin employed in treating gangrene usually consists of pooled, concentrated immune globulins against toxins of *Cl. perfringens, Cl. novyi,* and *Cl. septicum.*

8-10. **General Characteristics.** Members of the genus *Clostridium* are large, Gram-positive rods of variable length and breadth, ranging from long filamentous forms to short plump bacilli. In an appropriate environment, most species produce a single, round or oval spore, which may be located centrally, subterminally, or terminally within the vegetative cell. The spores appear as swollen bodies since they are generally wider than the diameter of the rods in which they develop. The shape and position of the spore, as well as the fact that it does, or does not, distort the vegetative cell are characteristics which help in species identification.

8-11. Spores of clostridia are not stained by the routine analine dyes. In Gram and methylene blue stained smears, spores are seen as unstained areas against the darkly staining cytoplasm, or as free hyaline bodies shown in figure 10. The relatively impervious spores may be effectively stained by the Wirtz-Conklin technique discussed in Chapter 4 of Volume 1. Stained smears of culture materials usually reveal spores, except that *Cl. perfringens* fails to sporulate on most laboratory media.

8-12. Gram-stained smears of fresh clinical specimens show large Gram-positive rods with or without spores. Unfortunately, however, the bacilli of gas gangrene cannot be distinguished morphologically from the saprophytic putrefactive anaerobes that may be associated with gangrene. Frequently, specimens from gangrenous lesions are contaminated with Gram-negative rods and Gram-positive cocci. For these reasons, direct smears are only of presumptive value, and gangrenous lesions must be cultured. Direct examination of suspected foods from outbreaks of botulism is of little or no value, since very few organisms are ordinarily present in such specimens.

8-13. The clostridia are obligate anaerobes. The majority of them are motile, but *Cl. perfringens,* the species most frequently isolated from clinical material, is nonmotile. Growth may

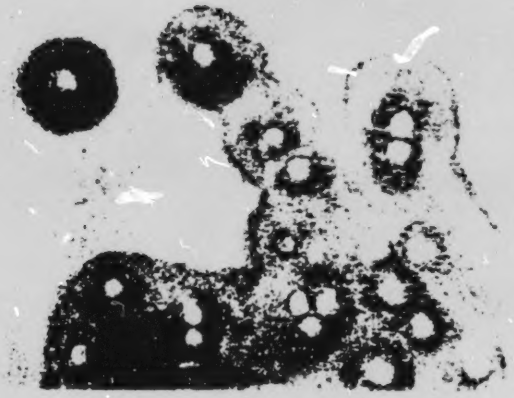


Figure 11. *Clostridium perfringens.*

be obtained over a wide range of temperature, but 37° C. is optimum for pathogenic species. Although the nutritive requirements among the clostridia vary, the organisms can be isolated from specimens using blood agar or thioglycollate medium containing 0.6 percent glucose. Anaerobic conditions may be provided by incubating the inoculated blood agar plates in a Brewer anaerobic jar. The clostridia grow well in the depths of thioglycollate medium, which provides the necessary anaerobic conditions without the necessity of providing an airtight seal for the mouth of the culture tube.

8-14. **Laboratory Identification.** On blood agar, after 48 hours anaerobic incubation at 37° C. typical colonies of almost all *Clostridium* spp. produce distinct beta hemolysis. *Clostridium perfringens*, however, may exhibit a "target" or double zone of hemolysis, as shown in figure 11. This is seen as a definite, narrow 1 to 2 mm. zone immediately around the colony, surrounded by a wider 4 to 5 mm. zone of partial hemolysis. If growth of large Gram-positive bacilli is obtained on blood agar in an anaerobic culture, a member of the genus *Clostridium* should be strongly suspected. On the other hand, if only thioglycollate medium is used and it yields Gram-positive organisms from the specimen, we must take one additional step. Inoculate some of the broth to two blood agar plates, and incubate one plate aerobically, the other anaerobically. Confirmation that the isolate is an obligate anaerobe is essential, since thioglycollate broth will support the growth of aerobic *Bacillus* spp. as well as the anaerobic *Clostridium* spp.—both Gram-positive rods.

8-15. In addition to clostridia, gangrenous infections may contain coliform bacteria, *Pseudomonas* spp. or *Proteus* spp. Under such circumstances, the primary anaerobic blood agar plate may be overgrown with these Gram-negative organisms, thereby making the isolation of *Clostridium* spp. on blood agar difficult or impossible. If overgrowth occurs, incubate the primary thioglycollate medium (inoculated at the time the plate was streaked) for 48 to 72 hours. During this extended period of incubation the Gram-negative bacilli will tend to die out, allowing isolation of clostridia in subculture.

8-16. When spores of a possible *Clostridium* spp. are seen in thioglycollate medium containing a mixed culture, heat resistance of the spore form may be used to advantage in obtaining isolation. An inoculum consisting of a sample of the mixed bacterial culture is placed in a fresh tube of thioglycollate medium and heated at 80° C. for 15 to 30 minutes. This heating will destroy all vegetative cells, but not the spores. The heated medium is then incubated for 24 to 48 hours, and the germinating spores give rise to a population of clostridia free of nonsporeforming species. Rarely will *Clostridium perfringens* be isolated by this method, however, because the bacterium generally fails to sporulate in culture.

8-17. Bacteria may be tentatively identified as members of the genus *Clostridium* on the basis of strict anaerobiosis, and characteristic microscopic and colony morphology. Final identification is dependent upon the results of biochemical studies and the demonstration of exotoxin production. In foldout 3 we note that species differentiation is based on fermentation of a number of carbohydrates, along with differences in proteolytic activity and reactions to metabolic tests we have discussed previously. Media inoculated to detect carbohydrate fermentation (usually a trypticase agar augmented by a sugar) must be examined for acid production at regular and frequent intervals between 8 and 24 hours. Otherwise, destruction of the indicator by certain clostridia may give erroneous results.

CHAPTER 2

The Gram-Negative Cocci and Coccoid Forms

THE BACTERIOLOGIST has long been acquainted with members of the family *Neisseriaceae*, the true Gram-negative cocci, because two species of the aerobic genus *Neisseria* have proved to be persistent challenges to the medical profession. *Neisseria gonorrhoeae*, the causative agent of the venereal disease, gonorrhea, is still prevalent around the world. The meningococcus, *N. meningitidis*, poses a constant threat in the form of outbreaks of cerebrospinal meningitis, even in the most advanced nations.

2. The other organisms of the family *Neisseriaceae*, the anaerobic *Veillonella* species, are not as well known, but these Gram-negative cocci are associated with disease as secondary invaders or as "opportunist pathogens." The bacteriologist must now also contend with three genera of cocculike (coccoid) organisms whose relationship to human disease has only been appreciated in recent years: *Mima, Herellea,* and *Moraxella*. The morphological similarity of these genera to the *Neisseria* and *Veillonella*, and the occurrence of each of these groups in clinical specimens, complicates the diagnostic problem.

3. The aforementioned coccoid forms are thought to be closely related taxonomically to the *Neisseria*, although no agreement has been reached as to their precise position in the classification scheme. For the present *Mima, Herellea,* and *Moraxella* species can be treated as a tribe within the family *Neisseriaceae*.

Order IV. *Eubacteriales*
  Family VIII. *Neisseriaceae*
    Genus I. *Neisseria*
    Genus II. *Veillonella*
  Tribe—*Mimeae*
    Genus I. *Mima*
    Genus II. *Herellea*
    Genus III. *Moraxella*

4. There is one last genus that we will mention in this chapter, the *Mycoplasma*, or so-called pleuro-pneumonia-like organisms (PPLO). Four species have been isolated from clinical materials originating in the alimentary and respiratory tracts and the genitalia. These Gram-negative forms are sufficiently different from the *Eubacteriales*, the true bacteria, that the PPLO have been set apart in their own order and family as indicated below.

Order X. *Mycoplasmatales*
  Family I. *Mycoplasmataceae*
    Genus I. *Mycoplasma*

9. Neisseria Infections of Man

9-1. Most of the ten *Neisseria* species are saprophytes, but two of them, *N. gonorrhoeae* and *N. meningitidis*, are human pathogens, as we noted previously. During an infection these two organisms are often seen intracellularly in white blood cells. The nonpathogenic *Neisseria* occur extracellularly and are important only in that they may be mistaken morphologically for pathogenic forms. Rarely have any of the saprophytes been implicated in disease. With the exception of *N. gonorrhoeae* and *N. cabiae*, all species may be encountered in the respiratory tract of normal individuals.

9-2. **Clinical Significance.** The gonococcus invades the mucous membranes of the genitourinary tract or the conjunctiva. Typical gonorrhea of males results in a urethritis characterized by painful urination and the formation of greenish-yellow pus. In gonorrhea of females, the untreated infection usually spreads from the vagina to the urethra, cervix, and rectum. Such infections give rise to a mucopurulent discharge. The gonococcus may invade the bloodstream from localized infections in both males and females, and lesions can form in the joints, heart valves, and meninges. *N. gonorrhoeae* commonly infects the eyes of the newborn (opthalmia neonatorium) during passage through the birth canal. If untreated, the infection usually results in permanent blindness. In the United States the incidence of gonorrheal conjunctivitis has been greatly reduced by the legal requirement that as a preventive measure suitable medications be instilled into the conjunctival sac of all newborn infants.

9-3. The portal of entry for meningococci is the nasopharynx. The organisms may constitute part of the transient flora in immune individuals, producing no symptoms, or they may set up a local nasopharyngeal infection in susceptible persons. The infection can extend to the bloodstream and to the meninges, causing meningitis. *N. meningitidis* has at least four recognized serologic groups based on antigenic differences in the cell. Since 1950, the alphabetical letters A, B, C, and D have been used to denote them. Group A strains are responsible for the majority of epidemics. Groups B and C have cause sporadic cases of meningitis, but Group D strains have rarely been isolated from the disease in the United States. The serological typing of *N. meningitidis* found in nose and throat cultures should be done, because an increase in Group A in a given population could have public health significance.

9-4. A number of saprophytic *Neisseria* species can be isolated from the upper respiratory tract of man. The most common are *N. catarrhalis*, *N. sicca*, and members of the pigmented flava group—*N. flavescens*, *N. subflava*, *N. flava*, and *N. perflava*. These organisms present some difficulty in laboratory diagnosis, for they must be distinguished from the pathogenic species, especially *N. meningitidis*.

9-5. **Laboratory Identification.** *Neisseria* species characteristically appear as diplococci, approximately 0.6 by 0.8 micron in size. The organisms are nonsporeforming and nonmotile. In stained smears of pus or body fluids the paired cells often have the shape of coffee beans or kidney beans, joined together on their concave or flattened sides. The organisms are seen within the polymorphonuclear leukocytes in detail A of foldout 4A. Leukocytes may contain 20 to 50 cocci, or more. In stained smears from cultures, the typically flattened border of adjacent cells is observed. Note, for example, the arrangement and morphology of diplococci in detail B of foldout 4A. While both detail A and detail B show *N. gonorrhoeae*, the microscopic morphology of *N. meningitidis* does not differ sufficiently to permit distinction between the two species.

9-6. Extra care must be taken in preparing smears from clinical materials containing these cocci. Swab specimens should be lightly rolled, rather than rubbed, on glass slides to accomplish an even distribution and to prevent destruction of white cells or tissue cells. This technique will give smears of even thickness that are easily scanned for the presence of intracellular organisms. The preparation of joint fluids for direct examination and culturing of *N. gonorrhoeae* is best accomplished by centrifuging the specimen and using the sediment as an inoculum. In the later, chronic stages of gonorrhea, few cocci are present in clinical materials.

9-7. Suspected cases of gonorrhea should be cultured whenever a presumptive diagnosis cannot be made by smear. In males, the urethral discharge is taken directly from the penal orifice. If no discharge is present, make a smear and culture the sediment centrifuged from the first 5 to 10 ml. of voided urine (the first morning specimen is preferred). It is more difficult to obtain a cultural diagnosis of gonorrhea in the female. The urethra and the cervix provide the most productive specimens; pus from the vagina ordinarily contains too many contaminating organisms. Since the gonococcus is relatively delicate and labile, inoculate the exudate directly from the patient to freshly prepared culture media.

9-8. In cases of meningococcal meningitis the spinal fluid usually contains polymorphonuclear white blood cells. Therefore, any cloudy or "milky" spinal fluid received by the laboratory is considered potentially infectious. We must take all necessary precautions, i.e., aseptic techniques, to insure the safety of laboratory workers. It is important to immediately inoculate spinal fluid and blood specimens taken from suspected cases. If prompt inoculation of pharyngeal swabs is not possible, the meningococci can be preserved for several hours by placing the swab in a sterile test tube containing a small amount of sterile horse blood.

9-9. An enriched medium is required for cultivation of *N. gonorrhoeae* and *N. meningitidis*. Those saprophytic species that are normal flora of the respiratory tract will grow on ordinary nutrient agar. A variety of media are available for isolation of the pathogenic forms, but chocolate agar is easily prepared and will give excellent results in routine use. We must be aware, however, that nutrients usually employed in preparing chocolate agar lack the special enrichment required for growth of the pathogenic *Neisseria*. For this reason, the chocolate agar base should be supplemented with yeast or liver extract to provide additional growth factors. The isolation of *N. gonorrhoeae* on blood agar is rarely accomplished because unheated blood is toxic to most strains. *N. meningitidis* will grow on blood agar, but chocolate agar increases the probability of isolation.

9-10. A newly developed selective medium (Thayer and Martin) for isolating gonococci and meningococci permits growth of those organisms while greatly suppressing saprophytic *Neisseria* species. Overgrowth of gonoccal colonies by bacterial contaminants present in cervical, vaginal,

TABLE 8
IDENTIFICATION OF *NEISSERIA* SPECIES

Species	Growth Characteristics	Carbohydrate Fermentation* CTA Medium				
		Glucose	Sucrose	Maltose	Levulose	Mannitol
N. gonorrhoeae	Enriched media required; growth 37°C. in CO_2. Small convex, smooth, glistening transparent, gray-white colonies with undulated margin. 0.5 to 1.0 mm. in diameter.	+	−	−	−	−
N. meningitidis	Enriched media required; growth 37°C. in CO_2. Smooth, mucoid, round, translucent, colorless, or gray-white colonies. 2 to 3 mm. in diameter.	+	−	+	−	−
N. catarrhalis	Grows on ordinary nutrient media at 22°C. and 37°C. Colonies on blood agar smooth, translucent, white with irregular margins or hard and opaque.	−	−	−	−	−
N. sicca	Grows on ordinary nutrient media at 22°C. and 37°C. Colonies on blood agar are gray-white, opaque, firm, dry, wrinkled. Colonies tend to crumble or move when probed.	+	+	+	+	−
N. haemolysans	Grows at 22°C. and 37°C. On blood agar colonies are smooth, gray-white. Growth is slow and delicate. Large zone of hemolysis 2nd or 3rd day.	+	+	+	+	−
N. flavescens	Similar to *N. meningitidis* but has yellow pigment on primary isolation.	−	−	−	−	−
N. subflava	Same as *N. flava*.	+	−	+	−	−
N. flava	Grows on ordinary nutrient agar at 22°C. and 37°C. Colonies on blood agar are small, circular, raised, opaque, slightly mucoid with yellow pigment.	+	−	+	+	−
N. perflava	Same as *N. flava*. This is the most common chromogenic *Neisseria*. Its presence in sputum may even color the sputum.	+	+	+	+	+
N. mucosa	Resembles *N. sicca* except colonies quite mucoid. Organisms are encapsulated.	+	+	+	−	−

* 48 to 72 hour reactions.

and rectal specimens is also reduced. *Mima polymorpha*, an organism we will discuss later in this chapter, fails to grow on this selective medium. *M. polymorpha* is easily confused with the gonococcus. The possibility of false positive cultural diagnosis of gonorrhea due to oxidase-positive *Mima* and saprophytic *Neisseria* is decreased by using the Thayer-Martin medium.

9-11. While *Neisseria* species are aerobic, an atmosphere of increased carbon dioxide—a candle jar—greatly favors colony development. The organisms also require a moist atmosphere which can be maintained by placing wet cotton or gauze in the bottom of the jar. The optimum growth temperature for pathogenic species, especially *N. gonorrhoeae*, is between 35° and 36° C.; however, a 37° C. incubator will give satisfactory results. After 48 hours incubation, pathogenic species appear as small, glistening, soft-textured, translucent or transparent colonies shown in detail C, *N. gonorrhoeae*, and detail D, *N. meningitidis*, in foldout 4A.

9-12. Four species of saprophytic *Neisseria* produce a yellow or yellow-greenish pigment: *N. flavescens*, *N. subflava*, and *N. perflava*. The other species found in man produce no noticeable pigment. Colonies are gray-white to tan in color. The *Neisseria* species can be differentiated from one another on the basis of growth characteristics and carbohydrate fermentation reactions in cystine-trypticase agar (CTA) summed up in table 8. A positive glucose coupled with negative reactions in the other carbohydrates points to *N. gonorrhoeae*. The meningococcus ferments both glucose and maltose, but the pathogen can be distinguished from *N. subflava* (which gives the same reactions) by colonial morphology and pigment production.

9-13. CTA is especially recommended for fermentation studies because *Neisseria* spp. will grow on it without an increased CO_2 atmosphere. Freedom from the CO_2 requirement is desirable, since absorption of the gas by fermentation media is accompanied by a drop in pH, resulting in false positive reactions (change in color of indicator). Moreover, CTA is a semisolid agar, which enables us to detect minute amounts of acid formed by weakly fermenting strains of *Neisseria*. Phenol red indicator is incorporated in the medium, so positive reactions are evidenced by a change from red to yellow within 18 to 72 hours.

9-14. The oxidase reaction is of particular value in examining an agar plate containing colonies of *Neisseria* spp. obscured by heavy growth of other organisms. Foldout 4A, detail E, shows the differentation of colonies by this technique.[1]

---

[1] **Bacteria that produce the respiratory enzyme, cytochrome oxidase, oxidize the reagent dimethyl-p-phenylene diamine, or the tetramethyl derivative, to give a color change that begins to appear within a few seconds. Oxidase-positive colonies turn pink, then maroon, and then black. Oxidase-negative forms are unchanged.**

TABLE 9

SPECIES DIFFERENTIATION OF THE GENUS *VEILLONELLA*

Organism	*Gas Produced Peptone Agar*	*Colony Morphology*	*Growth at* 22°C.	37°C.	*Indole*	*Gelatin*
V. parvula	+	Minute, transparent, bluish, weak hemolysis on blood agar.	Feeble	+	+	-
V. alcalescens	+	Minute, often a green pigment is produced. No hemolysis on blood agar.	±	+	-	-
V. discoides	+	(Deep colonies) Lenticular, up to 1 mm. in diameter. Grows in narrow disc 1 cm. below agar surface.	-	+	-	-
V. reniformis	-	(Deep colonies) punctiform then lenticular. 0.3 to 0.5 mm. in diameter.	-	+	+	-
V. orbiculus	-	(Deep colonies) Lenticular, large regular, whitish, translucent.	-	+	-	-
V. vulvovaginitidis	-	(Deep colonies) small, whitish.	-	+	-	+

Colonies treated with oxidase reagent will still give typical results on Gram staining. Identification of the genus *Neisseria* is established if the colony is oxidase-positive, and if it is found to be a typical Gram-negative diplococcus. Oxidase reactions cannot be used to distinguish species, however, since all *Neisseria* are oxidase-positive. Conformation of species is required where possible gonorrhea or meningitis is concerned. This may be accomplished by a careful study of growth characteristics and the reactions obtained in appropriate carbohydrate media as shown in table 8.

10. The Genus Veillonella

10-1. The anaerobic cocci occur in the mouth, respiratory tract, intestinal tract, and the urogenital system. Although *Bergey's Manual of Determinative Bacteriology* lists six distinct species, only one, *Veillonella alcalescens*, has been clearly characterized. Because of this, identification to species level is difficult in the clinical laboratory.

10-2. These organisms are small cocci measuring 0.3 to 0.4 micron in diameter, occasionally reaching a size of 2.0 microns. They occur in pairs, in short chains, and in masses. Sometimes individual cells cannot be distinguished within the masses of organisms. Colonial morphology is described in table 9.

10-3. Members of this genus are obligate anaerobes that grow well on routine bacteriological media. Optimum temperature is 37° C., but some strains grow at 22° C. Of the six *Veillonella* species, three produce gas in peptone agar, and three do not. Sugars are not fermented. The colonies are oxidase-negative. Laboratory diagnosis rests upon the morphology and biochemical activity of the genus and species set forth in table 9. The fact that these organisms are anaerobic and oxidase-negative differentiates them from *Neisseria*.

11. The Genera Mima, Herellea, and Moraxella

11-1. Members of the tribe Mimeae are found in the nose, vagina, and conjunctiva as saprophytes or as part of the normal flora. They have been recovered from many areas of the body as pathogens, however. These coccoid organisms (small, rounded bacilli) can cause conjunctivitis, meningitis, a gonorrhealike urethritis, and septicemia. They appear as secondary invaders in cases of severe burns. Organisms of the genera *Mima*, *Herellea*, and *Moraxella*, some of which are oxidase-positive and aerobic, are easily confused with *Neisseria* for that reason. The importance of distinguishing between them lies in the selection of antimicrobial agents, because many of the coccuslike forms, in contrast to *Neisseria* species, rapidly become resistant to antibiotics.

11-2. There are two recognized forms of *Mima*—*M. polymorpha* and *M. polymorpha* variety *oxidans*. These organisms have often been misinterpreted as gonococci in stained preparations. Generally, they appear coccoid in shape, but may be seen as rods, diplobacilli, or occasionally in short chains. The pleomorphic rods measure 0.5 to 0.8 micron wide by 1.0 to 3.0 microns in length. Diplococcal forms predominate on solid media—rods and filaments in liquid media. They are aerobic and usually nonhemolytic, although *Mima polymorpha* may exhibit a beta hemolysis on occasion. The major difference between *Mima polymorpha* and *Mima polymorpha* var. *oxidans* is that the latter is oxidase-positive.

11-3. The genus *Herellea* has one recognized species—*Herellea vaginicola*. This organism has been found as part of the normal skin flora in 25 percent of human males. It can be frequently isolated from feces, the normal vagina, and cases of vaginitis. *H. vaginicola* is encapsulated, measuring 0.5 to 0.8 micron in width and 1.0 to 3.0 microns in length. The organisms appear coccoid in shape, occasionally presenting a rodlike or filementous appearance. They are aerobic and generally nonhemolytic, although beta type hemolysis may be seen on occasion, as in the case of *Mima polymorpha*. *Herellea vaginicola* differs from the other organisms of the tribe Mimeae and the genus *Neisseria* in that it oxidizes xylose, while the others do not.

11-4. The five species of the genus *Moraxella* can be found in the same type of clinical material as *Mima* spp. and *Herellea vaginicola*. While the *Moraxella* resemble the *Neisseria* and may be confused with them, *Moraxella* spp. often show bacillary forms in a Gram-stained smear. Other morphological variations include diplococci and chains of coccoids.

11-5. In cultural and physiological characteristics, the tribe Mimeae is similar to the genus *Neisseria*. *Mima polymorpha*, *Mima polymorpha* var. *oxidans*, and *Herellea vaginicola* exhibit a luxuriant growth on chocolate or meat extract agar. Colonies are white, smooth, glistening, moist, and often viscid in appearance, as seen in figure 12. *Moraxella* colonies on blood agar appear translucent, gray-white, round, and viscid. Some degree of hemolysis may be seen. After 48 hours the colonies tend to become flattened. Organisms of the genera *Mima* and *Herellea* are often confused with certain of the enteric organisms because these two genera produce an alkaline-throughout reaction on triple sugar iron agar identical to that given by *Pseudomonas* and *Alcaligenes* species.

202

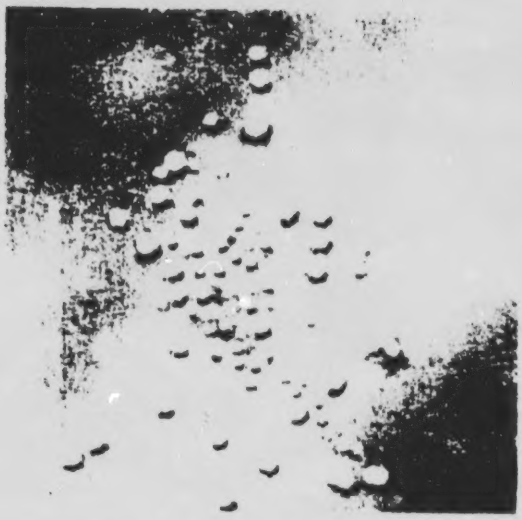


Figure 12. *Mima polymorpha.* Urine culture on chocolate agar.

11-6. In order to distinguish between *Mima polymorpha* and *Neisseria gonorrhoeae* (both are aerobic and oxidase-positive), urethral exudates should be inoculated to Thayer-Martin medium and MacConkey or eosin methylene blue (EMB) agar. Thayer-Martin contains inhibiting agents that retard the growth of *Mima* spp., *Herellea* spp., and many other bacteria which might be present in the exudate. MacConkey and EMB agar inhibit the growth of most *Neisseria* and allow the *Mima* and *Herellea* spp. and most enteric organisms which might be present to develop. Differentiation between the genera *Mima*, *Herellea*, and *Moraxella* rests mainly on carbohydrate fermentation studies, but in foldout 5 we see how a combination of laboratory tests can yield tentative separation of the cocci and coccoid organisms.

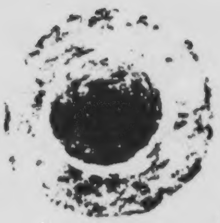


Figure 13. *Mycoplasma pharyngis.* Typical colony on agar.

12. The Pleuro-Pneumonia-Like Organisms (PPLO)

12-1. Four of the 15 known species of *Mycoplasma* have been found in clinical specimens obtained from the mouth and pharynx; the genitalia in cases of nonspecific urethritis; and the lungs in "atypical pneumonia." They have also been implicated in such diseases as chronic vulvitis, vaginitis, cystitis, and ovarian abscesses. *Mycoplasma* can reach the bloodstream as a result of trauma in childbirth, and it has been suggested that these PPLO's can be transmitted through sexual contact. Cases of infection traced directly to improper handling of clinical specimens in the laboratory have been reported.

12-2. These organisms are Gram-negative, but because the cell wall is poorly defined or absent, special staining procedures are needed. In size, the *Mycoplasma* lie between the bacteria and the rickettsiae. The average cell is approximately 125 millimicrons, although some cells grow as large as 250 millimicrons in greatest dimension. They may occur singly, in pairs, or in short chains. Because of the lack of a rigid wall, the cells may be quite pleomorphic, with ring, spiral, filamentous, and granular forms. Single cells of average size are below the resolving power of the conventional light microscope.

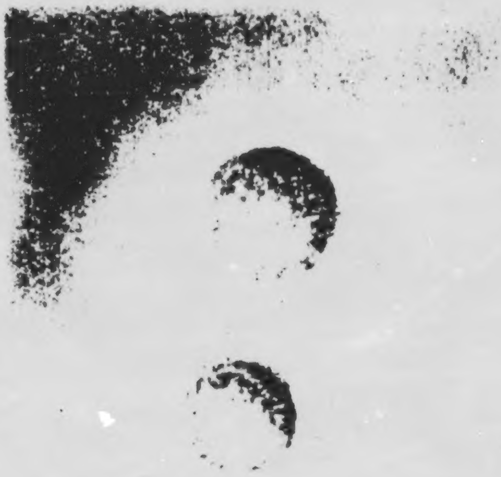


Figure 14. *Mycoplasma pneumoniae.* The Eaton agent.

12-3. The *Mycoplasma* are generally considered to be aerobic and facultatively anaerobic.

213

Media for laboratory cultivation must contain 20 to 30 percent ascitic fluid or 10 percent horse serum to supply unknown but necessary factors. Beta hemolysis is produced, but only on agar containing guinea pig red cells. Since the organisms grow into the medium, a soft agar is recommended. An alkaline pH (7.6 to 7.8) favors growth, and the optimum temperature is 37° C.

12-4. The organisms of the PPLO group cannot be studied morphologically by conventional staining techniques. Recognition is based on colony characteristics. The colonies (15 to 500 microns in diameter) are quite small, so the low power objective of the microscope is needed. After clinical material has been streaked to plates (PPLO media are available commercially) and incubated at 37° C. for 48 to 96 hours, colonies of *Mycoplasma* appear as small, round bodies with a thick center and have the appearance of a fried egg, as shown in figure 13. Some colonies appear granular and opaque, with a brown or yellow center. These are the so-called "mulberry" colonies, seen in figure 14. Both types of colony grow into the medium and are difficult to remove even with a wire loop.

12-5. Identification of colonies can be aided by using Dienes' staining method. We cut out a small block of the medium in which the suspected colony is growing and place it on a glass microscope slide, colony side up. A cover slip holding a drop of Dienes' stain (previously dried) is laid on the surface of the block, with the stained side in contact with the colony on the agar. The colonies are examined with the low power objective. They will show a dark blue center and light edges, as in figure 13. It is important to note that the stain is not decolorized by PPLO colonies but it is decolorized by bacterial colonies. Decolorization usually takes place in about 30 minutes.

The Small Gram-Negative Bacilli

THE SMALL GRAM-NEGATIVE bacilli are distinguished morphologically from the Gram-negative cocci and coccoid organisms by the tendency of the bacilli to grow in rod form. In many instances, however, the distinction is difficult to make. Under the microscope, a coccus, a coccoid form, and an oval-shaped short rod can look pretty much alike. We are quite often dependent, therefore, on knowledge of the source of the specimen and the clinical findings to supplement information gathered from morphological, cultural, and serological studies of the pathogen.

2. The Gram-negative bacilli, excepting the enteric organisms which will be discussed in the next chapter, are grouped together in two families, the Brucellaceae and the Bacteroidaceae. The first of these offers a wide variety of pathogens, ranging from the plague, tularemia, and undulant fever agents which are primarily animal parasites, to the *Hemophilus* species that cause whooping cough and chancroid, a venereal infection, in man. The family Bacteroidaceae is made up of five genera whose diverse membership includes minute, filterable forms as well as long, filamentous branching organisms. For the most part, they are inhabitants of the mucous membranes of warm-blooded animals and man.

3. This coverage of the small, Gram-negative bacilli will exclude many of the species that contribute only occasionally to disease. Our main emphasis will be placed on those microorganisms which one can expect to encounter with reasonable frequency in the clinical or epidemiological laboratory. We will touch briefly, however, on a few infectious agents representing genera that the bacteriologist should be familiar with, in general, even though he does not often find them among his cultures.

4. The taxonomic relationship of the small, Gram-negative rods we will mention is shown in the following scheme.[2]

Order IV. *Eubacteriales*

- Family V. *Brucellaceae*
  - Genus I. *Pasteurella*
  - Genus II. *Bordetella*
  - Genus III. *Brucella*
  - Genus IV. *Hemophilus*
  - Genus V. *Actinobacillus*
  - Genus VI. *Calymmatobacterium*
- Family VI. *Bacteroidaceae*
  - Genus I. *Bacteroides*
  - Genus II. *Fusobacrerium*
  - Genus V. *Streptobacillus*

13. Pasteurella and Francisella

13-1. Perhaps the best known pathogen of these closely related genera is *Pasteurella pestis* (now often referred to as *Yersinia pestis*), the etiologic agent of plague. Plague is primarily a disease of rodents which is secondarily transmitted to man. The wild rat is the usual host, but ground squirrels are also frequently infected. Plague is spread among rodents through the bite of fleas previously infected by a blood meal from a diseased animal. Human plague results when an infected flea feeds on man.

13-2. *Pasteurella pseudotuberculosis* is prevalent in soil, water, milk, and animal fodder. It causes a tubercular-type infection in a wide variety of animals. The agent has been isolated from human blood, spleen, liver, gallbladder, and spinal fluid. Symptoms resemble those of the enteric fevers, tularemia, or tuberculosis. *Pasteurella multocida* causes a hemorrhagic septicemia in warm-blooded animals, many of which harbor the organism on their mucous membranes. Infections in man can arise from the bite of domestic animals.

13-3. Until recent years *Francisella tularensis* was placed among the *Pasteurella* species as *P. tularensis*. It is the causative organism of tularemia or "rabbit fever" which, like plague, is primarily a disease of rodents secondarily acquired by man. The reservoir of infective agent is maintained in rodents by biting flies, ticks,

---

[2] This classification is employed in the latest edition (1957) of *Bergey's Manual of Determinative Bacteriology*. Since publication of the seventh edition, another genus, *Francisella*, has been established in the family Brucellaceae. This genus contains *Francisella tularensis*, formerly *Pasteurella tularensis*. In this chapter, we will use the newer designation, *Francisella*.

Table 10
Differentiation of *PASTEURELLA* Species

Species	*Hydrogen Sulfide Production*	*Indole Production*	*Nitrate Reduction*	*Growth on Urea Agar*	*Growth on Desoxycholate-Citrate Agar*
*P. pestis**	- (usually)	-	+ **variable**	No change in medium	Slight growth of reddish, pinpoint colonies; medium remains pink.
P. pseudotuberculosis	+ —	-	+	Colors medium red	Abundant growth of large, opaque yellowish colonies with medium turning yellow.
P. multocida	+	+	+	No change	No growth.

*Known also as *Yersinia pestis.*

and rabbit louse. All of these vectors are capable of spreading the disease from animal to animal. Humans contract tularemia either by handling the flesh of infected animals or through the bite of an arthropod vector. The main source of infection is the wild rabbit. The bacilli enter through cutaneous abrasions, or possibly through the intact skin of the hunter as he processes the animal for food. An aerosol of body fluids from infected animals may cause infection of the conjunctiva or lungs.

13-4. **Pasteurella Species.** Following entry of the plague bacillus into the body, the organisms spread by way of the lymph channels. The nodes become inflamed, hemorrhagic, and greatly enlarged, forming buboes in the groin or axilla—the so-called "bubonic plague." In many cases the bacilli spread via the bloodstream to the spleen, liver, and lungs. The septicemic phase is sometimes accompanied by subcutaneous hemorrhages which cause the formation of dark spots in the skin (black plague). Since bubonic infections may involve the lungs, individuals can disseminate plague to the respiratory tract of other persons (pneumonic plague) by coughing or sneezing a highly infectious aerosol.

13-5. When plague is suspected, material aspirated from buboes or sputum should be collected by strict aseptic technique. Blood specimens taken when the fever is high should be cultured periodically in the course of the infection. At autopsy, materials from lesions and inflammatory areas of internal organs, especially the spleen, will yield the organism. *P. pseudotuberculosis* gains entrance through breaks in the skin as well as the natural portals. The abdominal viscera is the site principally attacked, particularly in animals. Lesions of the cervical and thoracic lymph nodes or a septicemia can develop. Blood and diseased tissue specimens contain the infective agent. *P. multocida* is similarly isolated from inflamed areas or abscesses, especially those resulting from the bite of an animal.

13-6. Collectively, the three *Pasteurella* species appear as short, rounded rods or coccoids exemplified in foldout 4A, detail F. *P. multocida* is usually the smallest, ranging from 0.15 to 0.25 micron in width and 0.3 to 1.25 microns in length. *P. pestis* cells are intermediate in size, while *P. pseudotuberculosis* may reach 0.8 to 2.0 microns by 1.5 to 6.0. Bipolar staining is a frequent but not consistent property. Cells of each species can occur singly, in clusters, in pairs, or in short chains.

13-7. The *Pasteurella* organisms are facultative anaerobes that grow well at 37° C. or lower temperatures on routine culture media. For initial isolation, however, blood agar or an enriched medium such as trypticase soy agar is preferred. Colonies tend to be small (less than 1 mm. in diameter), round, glistening, grayish-white, and transparent, without evidence of hemolysis. A transition from mucoid to smooth or smooth to rough colony texture can sometimes be seen.

13-8. We distinguish among the three species by the criteria shown in table 10. Urease production and the inhibitory effect of bile salts revealed by growth characteristics on desoxycholate-citrate agar are the key points in this determination. The fact that *P. pseudotuberculosis* yields more abundant growth at room temperature than at 37° C. is helpful in differentiating this organism from the plague bacillus.

13-9. **Francisella Tularensis.** From our knowledge that the tularemia pathogen is closely related to the plague agent, we might expect similarities in symptoms, pathology, and the type of specimen we collect. These similarities do exist to a degree. Following invasion of the skin or mucous membranes, a papule or ulcer usually develops at the site of entry. The tularemia bacilli spread rapidly to the regional lymph nodes. A transient bacteremia during the first week of illness serves to distribute the organisms to various internal organs where foci of infection appear. As the disease progresses, pneumonia and a fulminating septicemia may develop. In some cases, there are no signs of localized involvement, but only the picture of a febrile systemic illness.

13-10. *Fr. tularensis* will not grow on ordinary media. Cultivation is most successful on cystine-glucose-blood agar containing thiamine, and growth is favored by incubation at 37° C. in a candle jar. Blood cultures utilize thioglycollate-heart-infusion agar or hemoglobin-cystine agar. Primary growth from the specimens requires from 4 to 7 days. In young cultures the colonies are very tiny, although later a relatively heavy growth of small, gray, transparent to translucent, mucoid colonies develop. Subcultures to cystine-glucose-blood agar plates usually yield abundant growth within 2 to 3 days. Lymph fluid, blood, sputum, and material from localized lesions in suspected cases of tularemia should be cultured. Blood samples may be taken repeatedly, particularly during the first week of infection. As in the case of the plague bacillus, strict aseptic technique must be maintained in handling tularemia cultures because of their infectivity.

13-11. The cells of *Francisella tularensis* measure 0.3 to 0.7 micron in length and about 0.2 micron in width. In young cultures the coccoid forms predominate, while in older cultures the bacillary form is more prevalent. The bacillary forms are usually quite pleomorphic and may be bipolar stained. Oval, bean shapes, dumbbell shapes, involution forms, and filamentous forms may be observed. Although this organism has no capsule, an envelope can been seen on a Gram-stained smear of exudates.

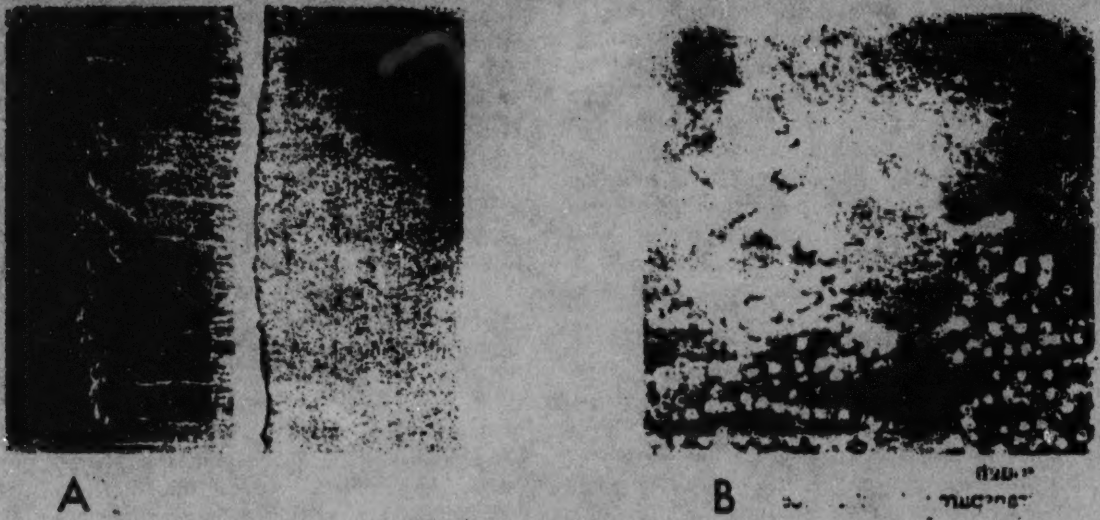
13-12. While biochemical tests have some value (acid production in maltose, glucose, and mannose), agglutination tests and animal virulence studies in mice and guinea pigs are more frequently used in confirming identification. Absence of growth on routine culture media serves to distinguish *Fr. tularensis* from the *Pasteurella* species.

14. Hemophilus and Bordetella

14-1. It was only recently that three species of *Hemophilus* were split off from that genus and placed in a new group, the *Bordetella*. Members of these two genera are morphologically similar, and they are found, with a few important exceptions, in the same habitat—the human

TABLE 11
SOURCE OF *HEMOPHILUS* SPECIES

Species	Source
H. influenzae	Nose, ear, and throat infections; pneumonitis; meningitis; septicemia.
H. aegyptius	Contagious conjunctivitis (pink eye).
H. ducreyi	Genital chancroid (soft chancre).
H. hemolyticus	Nasopharyngeal infections.
H. parainfluenzae	Normal throat flora.
H. parahemolyticus	Normal throat flora.
H. vaginalis	Vaginitis.



A. SATELLITISM WITH STAPHYLOCOCCI ON BLOOD AGAR B. CULTURE OF SPINAL FLUID ON CHOCOLATE AGAR

Figure 15. *Hemophilus influenzae.*

nasopharynx and adjacent areas of the respiratory tract.

14-2. The taxonomic relocation of the *Bordetella* was justified by two features. First, the three species that were formerly called *Hemophilus* are antigenically related to each other; and, secondly, these species have basically different nutritional needs from the *Hemophilus* with regard to components of blood. For growth on laboratory media, *Hemophilus* species must have one or the other, or both, of two compounds: hemin (the X factor), a heat stable derivative of hemoglobin; and phospho-pyridine nucleotide (the V factor), a heat-sensitive respiratory coenzyme. *Bordetella* species do not require that these factors be supplied in the medium.

14-3. **Hemophilus Species.** Of the 15 or so different forms in this genus, 11 are associated with the respiratory tract or conjunctiva and 3 with the genital region. Table 11 lists the 7 species of interest to the laboratory technician and indicates the environment in which they are generally encountered.

14-4. As a rule, the *Hemophilus* forms are coccobacillary in shape, measuring about 0.3 by 1.5 microns, but *H. influenzae* tends to produce long, thin filaments and aberrant cells shown in foldout 4A, detail G. Capsules often appear in smears from young cultures. The source of the isolate sometimes influences its morphology; for example, a diplococcal form predominates in smears from spinal, synovial, or pleural fluid.

14-5. While some variation exists in the ability of *Hemophilus* to grow on human blood, as a rule most human blood is satisfactory. Rabbit blood may be substituted for the human product, but sheep blood contains substances that inhibit the growth of certain species and should only be used for the preparation of chocolate agar. These inhibitory compounds are destroyed by the temperature of 70° to 75° C. required to lyse red blood cells in making chocolate agar. Since heat-labile factor V is also destroyed in the process, the growth substance must be restored by adding commercially available enrichment supplements or yeast extract to blood agar after converting it by heat to chocolate agar.

14-6. If enrichment supplements are not available, a plain blood agar plate can be streaked with the specimen presumed to hold *Hemophilus* spp. Immediately thereafter, one or two streaks of a staphylococcus culture are applied at right angles to the primary inoculum. After 24 to 48 hours incubation, *H. influenzae* and other species requiring V factor will appear as small colonies growing in close proximity to the staphylococci. This phenomenon is referred to as "satellitism" and results from V factor production by the staphylococci. We can see in figure 15A how diffusion of V factor into the surrounding medium provides a readily available enrichment source for the *Hemophilus* spp. Normally, however, *H. influenzae* grows very poorly on blood agar, and supplemented chocolate agar is preferred for isolating these organisms. Plain chocolate agar is unsatisfactory unless growth supplements are added.

14-7. Blood cultures are best collected in trypticase soy broth and thioglycollate medium under increased CO_2 tension and then incubated at

37° C. Once growth is observed, aliquots are subcultured to rabbit blood agar or to freshly prepared, supplemented chocolate agar in a candle jar. Material such as spinal fluid sediment, sputum, eye swabs, and nasopharyngeal or sinus swabs should be planted directly on rabbit blood or supplemented chocolate agar. In figure 15B we show colonies of *H. influenzae* on chocolate agar.

14-8. *H. influenzae* strains isolated from most pathologic sources occur in the smooth (S) form, although both mucoid (M) and rough (R) colonies are found. The S colonies are raised, slimy, confluent, and nonhemolytic. They are about 2 to 4 mm. in diameter. M colonies are more mucoid in appearance, and microscopic examination will reveal the presence of capsules. The rough (R) forms are smaller, nonhemolytic, transparent to translucent colonies that present a discrete dewdrop appearance. The cells of R colonies are nonencapsulated. Colony morphology, alone, is not sufficient to distinguish between *H., influenzae* from the throat and *H. aegyptius* of pink eye.

14-9. *Hemophilus ducreyi* deserves mention because of the special technique employed in collecting and planting the specimen. This species is the etiologic agent of a venereal disease characterized by the formation of ragged, soft ulcers in the genital region. The ulcers, called soft chancres or chancroids, differ from the typical hard chancre of primary syphilis. The regional lymph nodes of the groin may become secondarily infected, and these buboes can rupture and form secondary ulcers. Chancroids often become infected with other bacteria.

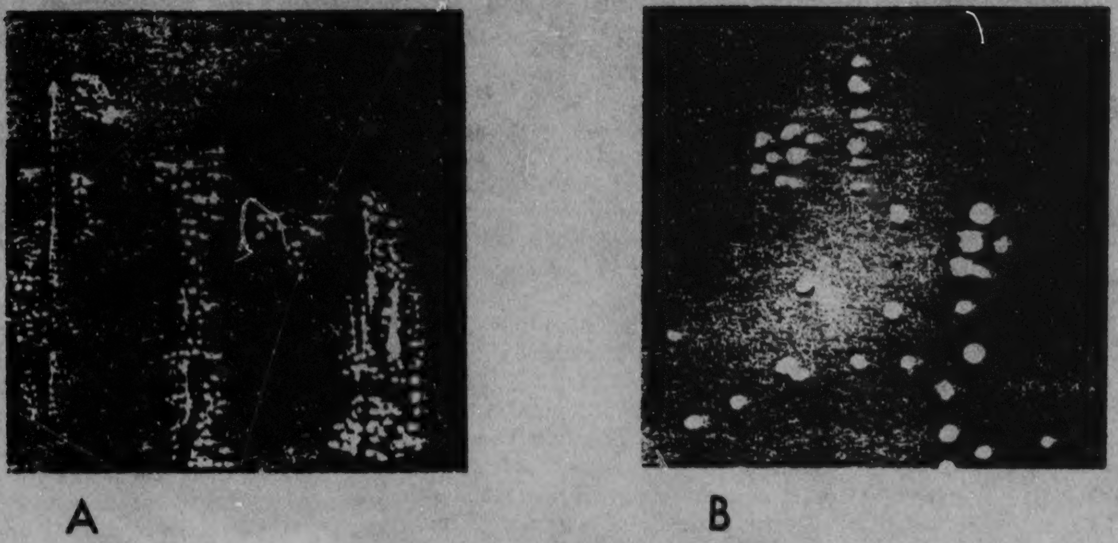
14-10. When observed in a smear of tissue exudates, the organisms appear as short ovoid bacilli in end-to-end pairs—diplobacilli—or short chains. The cells may be found in small clusters and in parallel rows—pallisades—presenting a "school of fish" appearance. Individual cells are about 1.0 to 1.5 microns in length and 0.6 micron in width.

14-11. Laboratory diagnosis is accomplished by taking material from beneath the craterlike edges of a suspicious lesion, and inoculating clotted rabbit blood or a meat infusion medium containing 3 percent agar and 30 percent sterile, defribinated rabbit blood. Incubation in a candle jar at 37° C. hastens growth. On the meat infusion agar-rabbit blood medium, the organism appears as small, convex, gray-white colonies with entire edges. In 2 or 3 days the colonies may measure 1.5 to 2.2 mm. in diameter and demonstrate a central depression. Whereas the pus aspirated from an unopened bubo may yield a pure culture, growth is obtained more frequently from the ulcerated lesion. We saw in table 11 that as many as 5 species of *Hemophilus* can be found in the same general locale—the

TABLE 12

GROWTH REQUIREMENTS AND HEMOLYTIC ACTION OF *HEMOPHILUS* SPECIES

Species	*X Factor*	*V Factor*	*Hemolysis*
H. influenzae	+	+	-
H. aegyptius	+	+	-
H. ducreyi	+	-	+
H. hemolyticus	+	+	+
H. parainfluenzae	-	+	±
H. parahemolyticus	-	+	+
H. vaginalis	-	-	+



A. THROAT CULTURE ON BORDET-GENGOU MEDIUM
B. SUBSULTURE ON BLOOD AGAR AFTER PRIMARY ISOLATION ON BORDET-GENGOU MEDIUM
Figure 16. *Bordetella pertussis.*

nasopharynx and nearby organs. Since these closely related forms are so similar in appearance, we generally separate them into species on the basis of requirements for X and V factors and the presence or absence of hemolysis on blood agar. In table 12 these variations in pattern are shown.

14-12. The site of the clinical infection must be depended upon to decide between *H. influenzae* and *H. aegyptius*, the two most prominent pathogens. Similarly, *H. parainfluenzae* and *H. parahemolyticus* can give identical cultural results, but distinction is not critical because both are normal flora of the throat.

14-13. **Bordetella Species.** We pointed out in the introductory paragraph of this section that three species, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were formerly classified with the genus *Hemophilus*. The *Bordetella* species are named for the French microbiologist, Jules Bordet, who isolated the whooping cough agent in 1906. The *Bordetella* are less demanding in their nutritional requirements with respect to the X and V factors of blood. This characteristic, we noted, was one of the reasons for classifying these organisms separately.

14-14. *Bordetella pertussis* is responsible for an infection of the trachial and bronchial epithelium, a disease known as whooping cough. The organism interferes with ciliary action and causes episodes of uncontrollable coughing accompanied by inspiratory whoops. It can also cause cerebral and pulmonary complications. *B. parapertussis* and *B. bronchiseptica*, the nonpathogens, can be isolated from the nasopharyngeal region by swabbing or cough plate technique.

14-15. Morphologically, *B. pertussis* is a small, ovoid, Gram-negative rod that measures 0.3 to 0.5 micron in width and 1.0 to 1.5 microns in length. It is nonsporeforming and nonmotile. In bronchial secretions the bacilli usually occur singly and in masses. Occasional diplobacilli may be observed. Chains are not formed in exudates but may be seen in broth culture. Smooth colonies usually yield encapsulated cells. The bacilli of *B. pertussis* closely resemble the coccobacillary forms of *Hemophilus influenzae;* however, *B. pertussis* does not ordinarily produce the threadlike filaments or other pleomorphic forms exhibited by *H. influenzae.*

14-16. *Bordetella pertussis* is an aerobe and requires a complex medium. Laboratory identification is based on the isolation of the organism from nasopharyngeal swab cultures or by the cough plate technique. The medium of Bordet and Gengou containing glycine, potato, and 15 percent blood is recommended. The cough plate technique involves holding the petri dish of Bordet-Gengou medium about 6 inches from the mouth of the patient and having the patient cough onto the exposed medium. The plate is incubated at 37° C. and examined after 3 to 5 days. Growth is slow. Colonies are small, dome-shaped, and possess a gray metallic luster resembling mercury drops or a bisected pearl, as we note in figure 16. A beta hemolysis is produced; however, the edge of the zone of hemolysis is fuzzy and indefinite because of the large concentration of blood used in the medium.

14-17. The isolation of *B. pertussis* is easier if we treat the cotton-tipped nasopharyngeal swab with penicillin. We do this by depositing a drop of penicillin (1,000 units) on the surface of the medium. Then we swirl the swab through the drug and streak the drug-specimen mixture with a wire loop. Penicillin inhibits contaminating organisms and permits growth of *B. pertussis* colonies. Penicillin (5 units per 10 ml.) can also be incorporated in the medium to retard contaminants.

14-18. As a rule, carbohydrates are not fermented by *B. pertussis*, although an occasional lactose and glucose fermentor may be encountered. Approximately 70 percent of the strains isolated are catalase-positive. We find in table 13 that citrates are not used as the sole source of carbon, and the species is urea-negative. In fact, citrate utilization, urease production, motility, and nitrate reduction are the prime means of distinguishing *B. pertussis* from the other two species. The brownish hemolysis surrounding *B. parapertussis* colonies is caused by a high concentration of copper-containing protein. We noted earlier that the "fuzzy" hemolysis of *B. pertussis* results from the high concentration of red cells in the Bordet-Gengou medium.

15. The Genus Brucella

15-1. The disease in man known as brucellosis or "undulant fever" can be caused by any one of three *Brucella* species that normally occur as pathogens of domestic animals. *Br. melitensis* (Malta fever) is associated with the disease in goats, *Br. abortus* with cattle (Bang's disease), and *Br. suis* with swine.

15-2. Disease in man follows the ingestion of raw milk and improperly cooked meat from infected animals or direct contact with contaminated animal tissue. The incidence of brucellosis is high among slaughter house attendants, veterinarians, sausage makers, butchers, dairymen, and similar occupational groups exposed to infected animals.

15-3. After entry into the human host, the organisms spread by way of lymph channels and the bloodstream to various organs including the liver, spleen, bone marrow, and other areas of the reticuloendothelial system. The organisms form multiple abscesses in these tissues, and osteomyelitis or meningitis may occur. At the height of infection, acute febrile episodes (undulant fever) result as organisms are intermittently released into the bloodstream. The bacilli remain dormant in deep tissues (chronic brucellosis), and relapses may occur.

15-4. These organisms are predominantly small coccobacillary forms ranging from 0.4 to 3.0 microns in length and 0.4 to 0.8 micron in width. In freshly isolated cultures they are encapsulated and form smooth or mucoid colonies. Gram-negative cells occur singly, in pairs, or in short chains. *Brucella* spp. do not possess flagella, so they are nonmotile. *Br. melitensis* usually produces coccal forms in exudates and tissue, whereas *Br. abortus* and *Br. suis* produce the bacillary form.

15-5. Blood taken during the febrile stage is the specimen of choice for isolating *Brucella* species. Lymph aspirations, biopsy materials, spinal fluid, or swab specimens of deep lesions may also be examined. The isolates require complex media for growth. Although many special media have been devised, trypticase soy broth

TABLE 13
CHARACTERISTICS OF THE *BORDETELLA* SPECIES

Species	*Citrate*	*Urea*	*Motility*	*Nitrate Reduced*	*Hemolysis*
B. pertussis	-	-	-	-	+ (fuzzy)
B. parapertussis	+	+	-	-	+ (brown)
B. bronchiseptica	+	+	+	(+)	+

() = usually

TABLE 14

IDENTIFICATION OF *BRUCELLA* SPECIES

Species	Carbon Dioxide Requirements	Carbohydrate fermentation			Growth in presence of		Motility	Urease 4 hrs.	Hydrogen Sulfide
		Glucose	Lactose	Sucrose	Basic Fuchsin	Thionin			
Brucella melitensis	Not required for growth	±	-	-	+	+	-	±	+ or - for 1 day
Brucella abortus	Required for growth (usually)	±	-	-	+	-	-	±	+ for 2 days
Brucella suis	Not required for growth	±	-	-	-	+	-	+	+ for 4 days

NOTE: 1. All cultures are incubated in a candle jar for 4 days except cultures for carbon dioxide differentiation.

2. When fermentation media are incubated in an atmosphere of increased carbon dioxide, an acid reaction results, regardless of whether or not the carbohydrate is fermented. Therefore, following incubation, such cultures must be allowed to stand at room temperature overnight before reading. This will allow dissipation of the absorbed carbon dioxide and subsequent reversion of the indicator to only that reaction produced by the organism.

212

and agar are generally used. *Br. abortus* can only be cultivated from clinical specimens in an atmosphere of 5 to 10 percent carbon dioxide. *Br. melitensis* and *Br. suis* will grow with or without increased carbon dioxide. Prolonged incubation (several weeks) at 37° C. is often necessary for initial isolation; on subculture, growth usually occurs within 3 or 4 days.

15-6. On trypticase soy agar, colonies are small, translucent, smooth, glistening, and blue-gray in color. In pour plates made with blood agar, colonies resemble those of the viridans (alpha) streptococci. *Brucella* species demonstrate a smooth (S) to rough (R) dissociation pattern. Mucoid (M) colony forms may also be encountered. They are round, flat, regular, and gray to reddish-yellow. The low-virulence R colonies generally yield long filamentous bacilli that autoagglutinate in normal saline solution. It is possible to distinguish the three colony types on the basis of color when the colonies are stained by an aqueous solution (1:2,000) of crystal violet and observed with a dissecting microscope or hand lens. The R colonies appear a deep violet red, the S colonies a blue-green, and the M type a light bluish-red.

15-7. Isolates presumed to be *Brucella* species are subjected to the cultural studies summarized in table 14. By inoculating trypticase soy agar slants and incubating them—one in a candle jar, the other in normal atmosphere—we can separate *Br. abortus* from *B. melitensis* and *Br. suis*. Motility and fermentation tests with glucose, lactose, and sucrose help to distinguish *Brucella* species from *Salmonella* species. The former are nonmotile and do not usually ferment those carbohydrates. *Salmonella* organisms are motile and will ferment at least one of them.

15-8. We can incorporate thionin (1:100,000 dilution) and basic fuchsin (1:100,000 dilution) into separate culture media to help differentiate the species on the basis of growth or no growth. Note also in table 14 that urea is hydrolyzed rather rapidly by *Br. suis* (within 4 hours) and very little or not at all in this same period of time by the other species. Sustained hydrogen sulfide production as measured by the lead acetate test is another means of differentiating the species. Serological techniques are helpful, as well. A significant rise in *Brucella* antibody titer in the patient's serum is indicative of infection. A blood specimen collected during the acute phase of the disease and another during the convalescent phase should be kept frozen and then tested simultaneously to demonstrate the increase in titer.

16. Miscellaneous Gram-Negative Bacilli

16-1. In this section we will make brief mention of three specific pathogens representing two genera of the family *Brucellaceae* and one genus of the family *Bacteroidaceae*. These infectious agents are *Actinobacillus mallei* and *Calymmatobacterium granulomatis* of the first-mentioned family, and *Streptobacillus moniliformis* of the bacteroid family. Lastly, we will take note of the heterogeneous organisms grouped under the genus *Bacteroides*. These species live in such close association with man, and in such profusion, that they are frequently found in clinical specimens as "secondary-invaders" of the disease process.

16-2. *Actinobacillus mallei* is the etiologic agent of glanders, a disease of horses occasionally transmitted to man and to other domestic animals. Entry into the body occurs through a scratch on the skin, an abrasion of the mucosa, or from inhaling contaminated moisture droplets. The latter source of infection is of particular importance in the laboratory. The clinical type of the disease is dependent on the mode of entry. Infection of the skin results in a nodule surrounded by swelling and an area of lymphangitis. Infection by way of the mucosa gives rise to lymphatic involvement, and pneumonitis can result from inhalation of the bacilli.

16-3. *Actinobacillus mallei* is a small Gram-negative rod approximately 0.3 to 0.5 micron in width by 2.0 to 4.0 microns in length. The cells show rounded ends and may occur singly, in pairs, or in groups. Filamentous forms are rarely seen, although branching cells may be found on glycine agar.

16-4. This organism grows aerobically and anaerobically with an optimum temperature of 37° C. It grows well on ordinary meat infusion media, but growth is more abundant if glycine and a small amount of glucose are added. On Loeffler's serum agar, the colonies appear moist, viscid, and yellow in color after 36 hours of incubation. On other media the colonies are round and granular, with the appearance of small yellow-white spots. Young colonies are transparent, becoming more opaque and more yellow with age. As a rule, no carbohydrates are fermented, and the organism is nonmotile, does not reduce nitrates, liquefy gelatin, nor produce indole.

16-5. It is best to inoculate material obtained from ulcerations and lesions to meat infusion agar and glycine agar. Growth occurs rapidly (24 to 48 hours) at 37° C. Inoculation to potato agar serves as a good diagnostic procedure in that there is good growth of a transparent, yellow-white, slimy layer on the agar surface. As

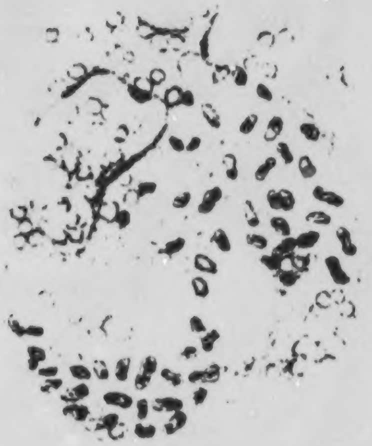


Figure 17. *Calymmatobacterium granulomatis.*

the culture gets older, the colonies become brown to red-brown in color. Blood cultures as a rule are negative but may become positive during the terminal stage of the disease.

16-6. *Calymmatobacterium granulomatis,* the only species of the genus, is the etiologic agent of granuloma inguinale, a disease characterized by chronic ulcerative lesions of the genital area. Although considered by many to be a venereal infection, transmission of the causative agent by sexual contact has not been fully substantiated.

16-7. The organism is a Gram-negative, pleomorphic rod exhibiting bipolar staining. The bacilli measure approximately 1.0 to 2.0 microns in length and have rounded ends. They occur singly or in clusters. Capsules may be observed when the organisms are found inside mononucleated white cells. When inside such cells, they are often referred to as "Donovani bodies," shown in figure 17. *C. granulomatis* cannot be isolated on ordinary culture media, but growth takes place in chick embryo tissues. Subculturing may then be successful on artificial media such as a beef-heart-infusion agar.

16-8. Laboratory diagnosis is accomplished by demonstrating "Donovani bodies" in large mononulcear white cells obtained from ulcerated lesions. If Wright's stain is used, blue bacilli surrounded by a well-defined, dense, pink capsule are observed. This organism, besides being morphologically similar to the enteric Gram-negative rods, demonstrates a cross-complement fixation with members of the genus *Klebsiella,* suggesting a closer relationship with the enteric forms than the taxonomic classification indicates.

16-9. *Streptobacillus moniliformis* is the one recognized species in the genus. This normal inhabitant of the mouth of rats, both wild and laboratory types, infects humans as the result of a rat bite. The disease is similar clinically to that produced by *Spirillum minus*—"ratbite fever." *Streptobacillus moniliformis* can be isolated from the blood. Collection of the patient's specimen in sterile 2.5 percent sodium citrate in the ratio of 10 ml of blood to 10 ml. of sodium citrate yields good results. The mixture should be centrifuged and the sedimented red cells used to inoculate culture media. Gram stains can be made from the sediment, too.

16-10. This Gram-negative organism is quite pleomorphic, varying in form from a short rod to long, interwoven filaments. These filaments break up into chains of bacilli and coccobacillary forms. Examination of the filaments often reveals yeastlike swellings. The filaments, which attain a length of 150 microns, may be curved and even looped. When cultured in a laboratory animal or on suitable media, a more uniform bacillary form measuring 2.0 to 4.0 microns in length emerges. The organism is nonsporeforming and nonencapsulated.

16-11. Blood, ascitic fluid, or serum is required for growth. Infusion media may be enriched by adding natural body fluids, e.g., serum, to a final concentration of 10 to 30 percent. This organism is facultatively anaerobic and grows well as an aerobe. It has an optimum temperature of 37° C. Some strains grow better in an atmosphere with a high moisture content. *Str. moniliformis* produces a colonial variant called "L_1" (Lister Institute). This L form grows into the medium and cannot usually be transferred with the laboratory loop. Transfer is accomplished by cutting out a small block of medium which contains a colony and then subculturing it to fresh media.

16-12. On solid media growth is slow—taking up to 48 to 72 hours. On serum or ascitic fluid media growth appears as discrete (1.0 to 2.5 mm. in diameter) colonies which are circular and colorless or gray-white. They are convex, with a smooth glistening surface that is butterlike in consistency. Coarse, granular colonies may be observed occasionally. L form colonies are found growing in the immediate proximity of the regular colonies. In broth media, growth usually occures at the bottom of the container. It resembles small white "fluff balls" or "cotton balls." If blood is used, growth on the sedimented red blood cells is quite striking. These "balls" can be removed intact for culturing and staining.

16-13. There are at least 30 different species in the genus *Bacteroides.* The organisms have been implicated in bacteremias, abscesses, peritonitis, salpingitis, meningitis, otitis media, and other conditions. But there is no direct evidence of a primary role for them in human disease. They appear to be associated with diseased or injured tissue as secondary invaders. The bacteroids are normal inhabitants of the mouth, intestinal tract, and vagina. In feces, they probably outnumber *Escherichia coli.* The type species of this genus, *Bacteroides fragilis,* and *Bacteroides funduliformis* are frequent isolates in the clinical laboratory. *Bacteroides melaninogenicus* has been found in surgical wounds and infections of the urinary tract.

16-14. The organisms of this genus are Gram-negative rods with a tendency to have rounded ends; however, one can see an occasional bacterium with pointed ends—fusiform. Some of these bacteroids are so small they can pass through the filter pads used in sterilizing liquids by filtration. Generally, the bacteria range in size from 0.3 to 0.8 micron in width and 2.0 to 4.0 microns in length. Filamentous forms as long as 100 microns may be observed occasionally.

16-15. The *Bacteroides* species are strict anaerobes, in many ways difficult to isolate because of their sensitivity to oxygen. Exposure to the air for just a few minutes causes death. Optimum temperature for growth is 37° C. Members of this genus ordinarily require a medium enriched with body fluids such as ascitic fluid, serum, or blood. Good growth can be obtained in thioglycollate broth plus ascitic fluid, and on blood agar. Colonies may take 48 hours to 10 days to become visible.

16-16. On blood agar, colonies of *Bacteroides* species are nonhemolytic, small, smooth, grayish in color, and glistening. *Bacteroides melaninogenicus* produces a melaninlike black pigment. This pigment may not show up before the fourth or fifth day, however. In broth culture, *Bacteroides* spp. produce a diffuse, even turbidity, a foul odor, and gas. Identity is established by the requirement for strict anaerobiosis, the Gram-negative microscopic morphology, and the characteristic colonies.

CHAPTER 4

The Enteric Organisms

OF ALL THE CLINICAL specimens submitted to the laboratory for bacteriological examination, the feces contain the greatest number of organisms, both in quantity and in the variety of genera represented. The feces normally contain millions of bacteria per gram. Most of these bacteria are harmless normal flora, but others cause some of the world's most prevalent diseases. The pathogenic and nonpathogenic Gram-negative bacilli that you isolate from stool specimens are often referred to as the "enterics" because of their natural habitation in the intestinal tract. Aside from these forms, there are "entericlike" organisms which closely resemble the enterics, and which occur often enough in feces or other clinical specimens to complicate the process of identification.

2. The prompt and accurate identification of enteric organisms serves several useful purposes. It can help locate and isolate "carriers" and give advance warning of epidemics. It can confirm the nature of an infection in cases where the clinical picture is not typical; and it can assist the physician in prescribing an effective course of therapy. The enterics are not limited to the feces, however. Almost every organ or body fluid may become diseased as a result of infection with these microbes. Consequently, learning the enteric identification procedures is an important part of the bacteriologist's training, because this knowledge is put to daily use in the hospital laboratory.

17. Classification and General Characteristics

17-1. The bacteria that make up the assorted enteric genera are closely related in the physiological sense. Their similarities in fundamental metabolic processes usually make generic distinctions difficult. To complicate the task of laboratory identification, we often find marked variability from culture to culture of a given species with respect to fermentation patterns and other traits useful in characterizing an organism. As a consequence, there is still a great deal of uncertainty regarding the exact taxonomic position of many of the Gram-negative enteric forms.

17-2. **Taxonomic Relationship.** From a glance at the classification scheme below we can see that most of the enterics fall within the order *Eubacteriales* and within one family, the Enterobacteriaceae. We will also be concerned briefly with a single genus, *Alcaligenes,* of the family Achromobacteraceae, because *Alcaligenes fecalis* is often associated with the mixed populations of the intestinal tract. The second order (Pseudomonadales) shown in our classification scheme has two genera of medical significance—*Pseudomonas* and *Vibrio. Ps. aeruginosa* and *Ps. pseudomallei* warrant special mention in this chapter although neither is normally associated with infections of the intestinal tract. The spiral-like *Vibrio cholerae,* causative agent of human cholera, represents the second genus of interest to us. It will round out our discussion of the enterics and related organisms.

17-3. The four families hold 37 genera. Fortunately, we are concerned in the clinical laboratory with less than a dozen of these:

Class II. Schizomycetes
Order IV. Eubacteriales
  Family III. Archromobacteraceae
    Genus I. *Alcaligenes*
  Family IV. Enterobacteriaceae
    Genus I. *Escherichia*
    Genus II. *Aerobacter*
    Genus III. *Klebsiella*
    Genus IV. *Paracolobactrum*
    Genus VII. *Serratia*
    Genus VIII. *Proteus*
    Genus IX. *Salmonella*
    Genus X. *Shigella*
Order I. Pseudomonadales
  Family IV. Pseudomonadaceae
    Genus I. *Pseudomonas*
  Family VII. Spirillaceae
    Genus I. *Vibrio*

TABLE 15
PRINCIPAL DIVISIONS AND GROUPS OF THE ENTEROBACTERIACEAE

IMViC Reaction	H_2S Production	Urease Production	Growth in KCN	Phenylalanine Deaminase Production	Division	Groups
V + - -	-	-	-	-	Shigella-Escherichia	Shigella Escherichia Alkalescens-Dispar
- + - +	+	-	V	-	Salmonella-Arizona-Citrobacter	Salmonella Arizona Citrobacter Bethesda-Ballerup
- - + +	- or late +	- or late +	+	-	Klebsiella-Aerobacter-Serratia	Klebsiella Aerobacter Hafnia Serratia
V + - V	V	V	+	+	Proteus-Providence	Proteus Providence

KEY:
+ = positive reaction
- = negative reaction
V = variable strains

* Adapted from P.R. Edwards and W.H. Ewing, Identification of Enterobacteriaceae, 2nd Edition, Burgess Publishing Co., Minneapolis, Minn. (1962), pp 2-3.

17-4. This nomenclature and taxonomic grouping conforms to the current edition (1957) of *Bergey's Manual of Determinative Bacteriology*. The system of Breed, Murray, and Smith, which served as the foundation for that edition with respect to the Enterobacteriaceae, grouped the enterics on the basis of lactose fermentation (positive or negative), pigment production, and the absence of cytochrome oxidase. The fermentation characteristic is not consistent, however, because each group of lactose-fermenting organisms contains strains which ferment the sugar slowly if at all, i.e., 48 or 72 hours instead of the usual 24-hour period. Moreover, pigmentation is variable and, as a rule, will not develop on media used routinely for enterics.

17-5. More recently, other workers have used IMVIC reactions (indole, methyl red, Voges-Proskauer, citrate), along with hydrogen sulfide and urease production, growth in KCN broth, and the production of phenylalanine deaminase to separate the principal enteric organisms into the "divisions" and "groups" portrayed in table 15. Note that emphasis is placed on a taxonomic aggregate (i.e., division) rather than the genus as the major element in classification.

17-6. Note, too, that the genus names represent a significant departure from the nomenclature (paragraph 17-3) used in *Bergey's Manual*. You will find examples of both the older and the more recent names in current reference texts and scientific periodicals. In this chapter we will use a format adapted from Edwards and Ewing (table 15) in expectation that the next edition of *Bergey's Manual*, tentatively scheduled for publication in 1969, will include these newer concepts of taxonomic relationship among the enteric forms.

17-7. **Morphology and Cultural Properties.** In the first volume of this course we discussed individually the steps usually followed by the bacteriologist in setting up isolation and identification procedures. Collection and preparation of stool specimens, and the physiological basis for employing the various types of culture media were discussed in Volume 1. We also gave you detailed instructions on how to inoculate specimens of different categories to specific media in order to successfully isolate the microorganisms ordinarily found in those specimens.

17-8. The processing of stool specimens for enteric isolation follows a well-established pattern. It is worthwhile to review the stepwise procedure in brief fashion. First, inoculate an enrichment broth (selenite F or tetrathionate). This procedure permits the enteric pathogens such as *Shigella* and some *Salmonella* spp. to multiply at the expense of the nonpathogens. After a few hours, streak the broth to obtain isolated colonies on differential media.

17-9. Concurrent with inoculation of the enrichment broth, streak plates of assorted selective and differential media with the stool specimen. Typical of the selective media are *Salmonella-Shigella* agar, bismuth sulfite, and desoxycholate. Selective media suppress growth of contaminating forms, usually species of nonpathogens, and allow the infectious species to survive and proliferate. Differential agars such as eosin-methylene blue, Endo's, and MacConkey's employ the principle of color distinction between lactose-positive and lactose-negative colonies (nonpathogens and pathogens). Colors are based on change in pH of an indicator dye when acids are produced from lactose.

17-10. Isolated colonies on these media furnish the inoculum for one of the more complex formulations such as triple sugar iron (TSI) or Kligler's agar slants. Reactions on TSI or Kligler's provide presumptive information on which we can base our choice of fermentation and other metabolic tests to establish genus and species identity. The three color patterns illustrated in foldout 4A, detail H, are possible with TSI or Kligler's agar:

- Acid (yellow) reaction throughout—lactose fermentation.
- Acid (yellow) butt and alkaline (red) slant—glucose fermentation.
- Alkaline (red) butt and slant—no sugars fermented.

17-11. In addition, gas formation is shown by bubbles in the medium, and H_2S production is evidenced by a blackening of the agar in the butt of the tube. TSI contains a third sugar, sucrose, which, if fermented, points to one of the slow-lactose fermenters. A positive sucrose gives an all-yellow reaction identical to that of lactose fermentation. Foldouts 6, 7, and 8 depict schematically the subsequent steps that help us pinpoint the identity of an unknown Gram-negative organism on TSI agar. We will refer to these foldouts again in emphasizing means of separating the individual genera of the enterics.

17-12. Microscopic morphology of the enteric and closely related forms is not particularly helpful in identification. Each can appear as short, plump rods, or coccoids, or occasionally as elongated filaments. Grouping occurs in clusters of single cells, pairs, and short chains. Size difference is not a reliable criterion because all of the species generally range from 0.4 to 1.0 micron in width and 1.0 to 3.0 microns in length. The cholera vibrio sometimes proves to be the exception by growing in "comma" form, or as curved, S-shaped, or ringlike cells.

17-13. Colony morphology is not ordinarily distinctive enough to enable us to identify a genus by sight. In fact, colonial characteristics of some of the common enterics are practically identical on representative selective and differential media. Foldout 4A, details I and J, demonstrate clearly the close resemblance of the two pathogens, *Shigella* and *Salmonella*, on EMB agar. Comparison of growth on Endo agar in foldout 4A, detail K, and foldout 4B, detail L, just as effectively shows the dissimilarity on differential media of lactose-positive and lactose-negative enterics.

17-14. Despite the fact that lactose fermentation is variable in many bacterial strains, this fermentation property is still used as presumptive evidence for separating enterics into pathogenic and nonpathogenic groupings. Colorless colonies indicate that the organism is a nonlactose fermenter and, therefore, possibly pathogenic. Keep in mind, though, that delayed lactose fermentation will also cause colorless colonies for the first 24 to 48 hours of incubation. Colored colonies (and acid-throughout reaction on TSI) generally indicate that the organism is a lactose fermenter, and, therefore, harmless when isolated from adults. Certain lactose fermenters can nevertheless be quite pathogenic in infants, causing a severe diarrhea.

17-15. The enterics grow well on simple media. (*Vibrio cholerae* is unique within the group in that this organism requires an alkaline pH.) The enterics are normally cultured aerobically, although most of them are also facultative anaerobes. They proliferate at room temperature and at 37° C. Motility is somewhat variable, none produce spores, and capsule formation is an inconsistent trait.

18. The Enterobacteriaceae

18-1. Referring again to table 15, we find that the family Enterobacteriaceae has four major taxonomic divisions, each divided into at least two separate groups. We will discuss the means of distinguishing these taxonomic elements in the laboratory and then take up selected species of the order Pseudomonadales in comparison.

18-2. **Shigella-Escherichia Division.** The *Shigella-Escherichia* division comprises two recognized genera, *Shigella* and *Escherichia*, along with a poorly defined group known as *Alkalescens-Dispar* (A-D). The A-D group was formerly included in the genus *Shigella* as *Sh. alkalescens* and *Sh. dispar* although the group is rarely, if ever, associated with infections of man. The A-D category contains nonmotile organisms that are essentially slow lactose-fermenting *E. coli* strains; yet they possess the serological typeability of *Shigella* species.

18-3. Table 15 shows us that the *Shigella*, *Escherichia*, and A-D forms can be segregated from other enteric divisions by the IMViC reactions, H_2S production, and other simple tests. Lactose fermentation (denoted by TSI reaction) and

TABLE 16
BOICHEMICAL REACTIONS OF *E. COLI, ALKALESCENS-DISPAR,* AND *SHIGELLA*

	T.S.I.												
	Butt	Slant	H_2S	Urea	Indole	Citrate	Motility	Lactose	Mannitol	Xylose	Rhamnose	Dulcitol	Sorbitol
E. coli	A	A	-	-	+	-	+	+	+	+/-	+/-	-/+	+/-
Alkalescens-Dispar	A	V	-	-	+	-	-	D	+	+	+/-	+/-	+/-
Shigella	A	-	-	-	V	-	-	-	+/-	-/+	-/+	-/+	+/-

KEY
- - = 90% or more of strains are negative
- + = 90% or more of strains are positive
- +/- = more than 50% of strains are positive
- -/+ = more than 50% of strains are negative
- V = Variable strains
- D = Delayed reaction (more than 24 hours)
- A = Acid reaction

motility are particularly useful in differentiating organisms within the division. Table 16 lists additional biochemical reactions that help verify the identity of taxonomic elements within the division. Polyvalent *Shigella* antisera are available against *Shigella* subgroups A, B, C, and D. A fifth antiserum is used for members of the A-D group. By using the five antisera it is possible to identify all but a few of the rarer shigellas, as well as the common representatives of the A-D group.

18-4. Shigellosis, or bacillary dysentary, is essentially an inflammation of the mucous membrane of the large intestine and terminal ileum which can lead to necrosis and superficial ulceration. Symptoms usually occur within 1 to 2 days following ingestion of contaminated food or drink. The intense irritation of the bowel is due to the release of an endotoxin during lysis of the *Shigella* cells. Infections with *Sh. dysenteriae*, serotype 1, are more severe because, in addition to the endotoxin, an exotoxin (neurotoxin) is produced. Infections with exotoxin-producing strains are relatively frequent in India, Japan, China, and other parts of Asia. Although some patients pass organisms in the stools for only a short period, others become chronic carriers and serve as a reservoir of infection.

18-5. The genus *Escherichia* shares membership with the *Shigella* spp. in one of the principal taxonomic divisions shown in table 15. *Escherichia coli* is probably the most abundant bacterium represented in the normal intestinal tract, with the possible exception of *Bacteroides* spp. *E. coli* and other enteric saprophytes can cause disease when introduced into tissues outside the intestine. These organisms frequently invade the biliary or urinary tract, and *E. coli* is one of the most common causes of cystitis. It has also been isolated from local infections such as conjunctivitis. A number

TABLE 17
REACTIONS ON CITRATE-MANNITOL AGAR

Organism	*Slant Color*	*Junction Color*	*Butt Color*
E. Coli	Yellow	Blue	Yellow
Shigella[*]	No change	No change	No change
Providence	Blue	Blue	No change
Salmonella, Arizona, and *Citrobacter*	Blue	Blue	Yellow

[*] Rarely a *Shigella* isolate will produce a yellow slant and butt, but none has so far produced a blue slant-butt junction.

TABLE 18

GROUP DIFFERENTIATION OF ENTEROBACTERIACEAE BY BIOCHEMICAL TESTS (REACTIONS OF TYPICAL 24-HOUR CULTURES)

	Shigella-Escherichia		*Salmonella-Arizona Citrobacter*				*Klebsiella-Enterobacter-Serratia*					*Proteus-Providence*				
									Hafnia			*Proteus*				
	Shigella	*Escherichia*	*Salmonella*	*Arizona*	*Citrobacter**	*Bartholomew (Edwardsiella tarda)*	*Klebsiella*	*Enterobacter*	*37°C*	*22°C*	*Serratia*	*vulgaris*	*mirabilis*	*morganii*	*rettgeri*	*Providence*
Indole	(-)	+	-	-	(-)	+	-	-	-	-	-	+	-	+	+	+
Methyl Red	+	+	+	+	+	+	-	-	+	-	-	+	-	+	+	+
Voges-Proskauer	-	-	-	-	-	-	+	+	V	+	+	-	+	-	-	-
Simmon's Citrate	-	-	+	+	+	-	+	+	V	+	+	(-)	(+)	-	+	+
H_2S (TSI Agar)	-	-	+	+	+	+	-	-	-	(+)	(-)	+	+	-	-	-
Urease	-	-	-	-	-	-	(+)	-	-	-	-	+	+	+	+	-
KCN	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+
Motility	-	+	+	+	+	+	-	+	V	+	+	+	+	+	+	+
Gelatin	-	-	-	(+)	-	-	-	(+)	-	-	+	+	+	-	-	-
Lysine Decarboxylase	-	+	+	+	-	+	+	V	+	+	+	-	-	-	-	-
Phenylalanine Deaminase	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+

KEY:
+ = Reaction, growth or production
- = Nonreactive or no change
() = Usual reaction, occasional species or strains to contrary
V = Some species or strains positive, some negative

NOTE:
Citrobacter (Escherichia freundii) includes Bethesda-Ballerup group.

of *E. coli* serotypes have been associated with infant diarrhea. This organism gains entrance to the intentinal tract of man shortly after birth and remains there throughout life.

18-6. The growth of *E. coli* on differential enteric media is so characteristic that we usually have no difficulty in recognizing it. The brick red color on Endo agar (foldout 4B, detail L) and the dark, metallic sheen on EMB agar seen in foldout 4B, detail M, are generally typical of lactose-positive *E. coli*. The IMViC reactions, negative H_2S, and motility substantiate the visual observation.

18-7. Occasionally, however, nonlactose fermenting strains of *E. coli* are confused with *Shigella* spp. and the A-D group. In the absence of diagnostic antisera, a biochemical test is available to identify *E. coli* specifically. Simmon's citrate agar and a citrate-mannitol agar are used in combination. A small loopful of the unknown is stabbed and streaked to a slant of citrate-mannitol agar. Without flaming the loop, a slant of Simmon's citrate agar is surface-streaked with the same inoculum. An alkaline reaction (blue) appearing in 24 to 48 hours at the junction of the acid slant and acid butt of the citrate-mannitol agar (together with no growth on the Simmon's citrate agar slant within the same incubation time) is diagnostic of *E. coli*. Table 17 shows typical 24-hour reactions on citrate-mannitol agar.

18-8. *E. coli* is ordinarily unable to utilize citrate as a sole source of energy, but it *can* break down mannitol. In this procedure the energy obtained from mannitol in citrate-mannitol agar enables the organism to attack the sodium citrate present in the medium. The positive (blue) reaction shown in table 17 results from a pH change to alkaline when the sodium is released to form sodium carbonate. *Shigella* species and members of the A-D group are unable to accomplish this reaction.

18-9. **Salmonella-Arizona-Citrobacter Division.** These organisms can be separated taxonomically from the *Shigella-Escherichia* division on the basis of IMViC reactions and hydrogen sulfide production as seen in table 15. Differentiation of *Salmonella-Arizona-Citrobacter* strains from the *Klebsiella-Aerobacter-Serratia* division rests on the MR-VP test, and to some extent on H_2S production and growth in KCN broth. Separation from the *Proteus-Providence* division is based mainly on the results of the urease and phenylalanine deaminase procedures. A more detailed comparison of biochemical reactions within the enterics is provided in table 18. This table will be useful as we point out distinctions between genera within a given taxonomic division.

18-10. All *Salmonella* spp. are potential pathogens. They produce such diseases as typhoid and the other enteric fevers, septicemia, and gastroenteritis. These infections originate from the ingestion of contaminated food or drink. Many such conditions are brought about as a result of food or beverages being contaminated by "carriers," i.e., persons who harbor the pathogens without showing clinical symptoms of disease. The detection of carriers is as important as the confirmation of suspected clinical cases. You may recall that Typhoid Mary, the historically notorious carrier, was implicated in the direct transmission of typhoid fever to approximately 1,300 persons. She was reportedly responsible for at least seven epidemics of the disease.

18-11. *Salmonella typhi (typhosa)* is the organism responsible for typhoid, while *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, and *S. sendai* are most often encountered in paratyphoid fever. *S. paratyphi A* and *S. paratyphi C* are only occasionally found in the United States. *S. sendai* is isolated primarily in the Orient. In enteric fevers, the pathogens invade the small intestine, spread through the intestinal lymphatics to the thoracic duct and enter the bloodstream. The resultant bacteremia distributes the infection to many organs, including the kidney, liver, and gallbladder. Blood cultures are usually positive only during the first and second week of infection.

18-12. Of the many *Salmonella* spp. that produce acute gastroenteritis in man, *S. typhimurium* is the most frequently incriminated. *S. enteriditis* is possibly the second most common cause. Both of these agents can be spread by rodents in their feces. *S. cholera-suis* has also been implicated in gastroenteritis but to a lesser extent. Symptoms of gastroenteritis result from the irritating action of acids and bacterial endotoxin upon the patient's intestinal mucosa. The acids are formed through fermentation of carbohydrates by the pathogen. Endotoxins are released during cellular lysis of bacteria. Outbreaks of gastroenteritis, often referred to as "food poisoning," are usually linked to the consumption of certain foods that serve as good bacterial culture media. Disease usually originates from food contamination by rodents, unsuspected subclinical cases, or healthy carriers who harbor the organisms in their intestine, gallbladder, or urinary tract.

18-13. Organisms of the genus *Arizona*, first described in 1939, are related serologically to the salmonellas. Isolated mostly from reptiles, they are being found with increasing frequency in man. The genus has but one well-defined species, *Arizona arizonae*, which has been isolated from cases of acute diarrhea and gastroenteritis

Figure 18. *Salmonella* **species. Culture on desoxycholate agar.**

arising from contaminated food. Organisms of this genus are similar to the salmonellas morphologically, serologically, and biochemically, except that *Arizona* strains generally ferment lactose slowly, whereas *Salmonella* species are lactose-negative. *Arizona* does not ferment dulcitol. Fermentation studies with lactose and dulcitol generally differentiate the two genera. The typical lactose-negative colonies of *Salmonella* are shown in figure 18.

18-14. The *Citrobacter* group, which closely resembles the *Salmonella* and *Arizona* organisms, includes strains of *Paracolobactrum intermedium*, commonly referred to as the Bethesda-Ballerup group. Citrobacter is lactose-positive like *E. coli;* but unlike the coliforms, *Citrobacter* grows on citrate and produces H_2S, as table 18 indicates. Generally speaking, the Bethesda-Ballerup group consists of slow-lactose fermenting strains of *Citrobacter*. Identification of these organisms follows the scheme used for *Citrobacter* in conjunction with serological typing. Bethesda-Ballerup polyvalent typing serum is available for this purpose. *Citrobacter* and Bethesda-Ballerup have been suspected as the etiologic agents of enteric diseases, but their role in producing pathological states has not been precisely determined.

18-15. The *Salmonella-Arizona-Citrobacter* division contains one other group, the so-called *Bartholemew* organisms, which since 1965 have been placed under the genus *Edwardsiella*. The principal species, *Edwardsiella tarda*, has been isolated from stools, blood, and urine, as well as from contaminated water supplies. Many of its biochemical reactions, H_2S production and lactose fermentation excepted, coincide with those of *E. coli*. At the same time, indole production and inability to use citrate are the biochemical features in table 18 that distinguish *Edwardsiella* from the salmonellas and *Arizona*.

18-16. In laboratory practice the bacteriologist ordinarily follows a sequence of procedures such as those depicted in foldouts 6 and 7 to separate members of the *Salmonella-Arizona-Citrobacter* division. His starting point is the TSI reaction because lactose fermentation, positive or negative, dictates which pattern of biochemical tests he will subsequently employ. Even so, the results are not always clear-cut and straightforward. Regard one of the slow lactose fermenters, *Arizona*, as an example. If the culture is read at 24 hours, the alkaline slant and acid butt will probably be seen on TSI (foldout 7) because lactose has not yet been broken down. Urease production, citrate, motility, indole, and xylose determinations are required to make the several necessary distinctions among the species of *Salmonella*, the *Bethesda-Ballerup* group, and others. If the TSI slant is read at 48 hours, the TSI reaction will probably be acid-throughout (foldout 6). In this case *Arizona* can be distinguished from *Citrobacter*, *E. coli*, and related organisms by H_2S and urease production and growth in KCN broth.

18-17. **Klebsiella-Enterobacter-Serratia Division.** Table 18 shows us that this taxonomic division differs from the other divisions of the enterics by the IMViC pattern, coupled with growth in the presence of KCN. Delayed production of urease and H_2S is also characteristic of some strains. Since members of this division are lactose fermenters, scrutiny of table 18 shows that only the positive KCN reaction with *Citrobacter* and certain *Proteus* strains—also lactose-positive—leaves room for discrepancies in identification. H_2S formation and production of phenylalanine deaminase will resolve the issue.

18-18. The genus *Klebsiella* has three species: *Klebsiella pneumoniae*, *Klebsiella ozaenae*, and *Klebsiella rhino-scleromatis*. Of these species, *K. pneumoniae* (Friedlander's bacillus) is most often encountered in the laboratory. It can be isolated from the upper respiratory and intestinal tract of about 5 percent of normal individuals. It is said to be responsible for approximately 2 percent of the bacterial pneumonias. The organism is found as a secondary invader in such diseases as bronchiectasis, tuberculosis, influenza, pleurisy, and pyelonephritis. *K. ozaenae* can be isolated in nasal conditions such as ozena and atrophic rhinitis. *K. rhinoscleromatis* occurs in nasal secretions from rhinoscleroma.

18-19. There is a disagreement among bacteriologists as to the exact classification of *Klebsiella* and *Aerobacter* species. Many authorities no longer recognize the genus *Aerobacter* and consider its nonmotile strains to be *Klebsiella pneumoniae*. The motile strains of *Aerobacter* are now classified in the genus *Enterobacter*. The *Hafnia* group is in reality a subgroup of *Enterobacter* with certain temperature-dependent variations in IMViC biochemical properties seen in table 18.

18-20. The genus *Serratia* is exemplified by *Serratia marcescens*, a saprophyte generally found in soil, water, milk, and food. *Serratia* has been implicated in epidemics within hospitals in much the same manner as hospital staphylococci. Burn cases are particularly susceptible to the organism. *Serratia* can also be a secondary invader in certain types of lung disease. Unlike the other enteric forms, colonies on agar are generally circular, thin, smooth, or rough, occasionally mucoid, and often pigmented (red, pink, magenta). In broth cultures growth may form a red ring at the surface, or a pigmented pellicle.

18-21. *Klebsiella* and *Enterobacter* ferment lactose to yield an entirely acid (yellow) reaction on TSI. While *Serratia* and *Hafnia* are late or irregularly positive lactose fermenters, their positive sucrose reaction will also cause TSI to appear acid throughout. In foldout 6 we find that H_2S production, methyl red and indole tests, motility, and urease production serve to differentiate those four groups. If variations in reactions occur, the fermentations shown in table 19 are useful in establishing identity.

18-22. **Proteus-Providence Division.** The genus *Proteus* contains highly motile bacteria known for their ability to decompose urea rapidly and to deaminate phenylalanine to phenylpyruvic acid. Lactose is not fermented, although sucrose-positive strains give an acid slant and butt in TSI agar. Of the four species listed in table 18, two species, *P. vulgaris* and *P. mirabilis*, produce H_2S rapidly and abundantly, liquefy gelatin, and swarm on moist agar media. The other species, *P. morganii* and *P. rettgeri*, lack these characteristics. Swarming is a result of active motility. It can be curtailed by using media lacking in sodium chloride, or media with a 6 percent agar content, or by adding 0.1 percent chloral hydrate to a medium. Foldout 4B, detail N, shows the difference in appearance between discrete *Proteus* colonies and swarming colonies. In some instances swarming species spread progressively as a surface film over the entire plate.

18-23. Although the four species of *Proteus* are primarily free-living organisms in water, soil, and sewage, they are frequently isolated from fecal specimens of normal individuals. *P. morganii* is responsible for cases of "summer diarrhea" in infants and children. *Proteus* spp. cause human infections when introduced into tissues other than the normal intestinal tract. In this connection, *Proteus* ranks next to *E. coli* as the etiological agent of cystitis. The organisms are encountered

TABLE 19
DIFFERENTIATION OF SERRATIA-KLEBSIELLA-ENTEROBACTER-HAFNIA
BY BIOCHEMICAL MEANS

	Serratia	*Klebsiella*	*Enterobacter*	*Hafnia*
Glycerol (gas)	-	+	±	+
Glucose (gas)	±	+	+	+
Gelatin	+	-	±	-
Sorbitol	+	+	+	-
Lysine	+	+	±	+
Ornithine	+	-	+	+
Arginine	-	-	±	-
Motility	+	-	+	+
Pigment (25°C)*	±	-	-	-

*At 25°C. pigment is usually red.

TABLE 20
BIOCHEMICAL REACTIONS ON SELLERS' DIFFERENTIAL AGAR

Gram-Negative organism	*Butt*	*Band*	*Slant*	*Gas N_2*
Alcaligenes fecalis	(Alk)	–	Alk	(+)
Pseudomonas aeruginosa	(Alk)	(Alk)	–	+
Herellea vaginicola	–	A	Alk	–
Mima polymorpha	–	–	Alk	–

KEY:

A = Acid reaction, yellow color
Alk = Alkaline reaction, blue color
+ = Production
– = No reaction, no change
() = Usual reaction, occasional strains to contrary

NOTE:

Sellers agar is green when prepared sterile, pH 6.7.

N_2 gas is manifested by bubbles in condensate and/or cracked agar.

P.*aeruginosa* is distinguished by U-V **fluorescence** of slant, proteolytic activity, and nitrate reduction.

H.*vaginicola* oxidizes glucose, forming the yellow band.

frequently in eye and ear infections and occasionally in pleurisy, peritonitis, and suppurative abscesses in many areas of the body. *Proteus* spp. are commonly associated with other bacteria in purulent wounds and may contribute to the severity of such infections.

18-24. The *Providence* group closely resembles the genus *Proteus*, but the latter organisms split urea, whereas the Providence group does not (see table 18). *Providence* is known as *Proteus inconstans* in the 7th edition of *Bergey's Manual*. Providence organisms are isolated from the same clinical sources as *Proteus* and can produce the same type of disease states, including gastroenteritis.

18-25. In table 18 the key to distinguishing the *Proteus-Providence* division from other enterics lies in urease and phenylalanine deaminase production. The *Proteus* spp. are phenylalanine deaminase-positive and urea-positive. The *Providence* organisms are urea-negative but closely resemble *Proteus* in all other respects. The rapid decomposition of urea to ammonia (1 to 4 hours) yields a highly alkaline environment which can be detected with a pH indicator dye. We see in foldout 4B, detail O, the distinctive positive reaction of *Proteus*.

18-26. We noted above that some strains of *Proteus* and *Providence* ferment sucrose, thus giving an acid slant in TSI agar. Identity of these strains can be worked out according to the steps in foldout 6. Ordinarily, however, glucose fermentation (and negative sucrose and lactose reactions) result in the typical alkaline slant and acid butt which provide the basis for steps outlined in foldout 7.

18-27. The *Proteus* forms possess a number of O (somatic) and H (flagellar) antigens. The X-19, X-2, and X-K strains contain a distinctive O antigen that is useful in diagnosing certain unrelated diseases serologically. For example, cells of the X-19 strain react with the serum of patients suffering from typhus fever. This phenomenon is called the Weil-Felix reaction. It stems from the fact that *Proteus* X-19 and *Rickettsia prowazeki*, the agent of typhus, have an antigen so nearly identical that antibodies formed against the rickettsia also react with *Proteus* cells to give an observable agglutination in the test tube.

18-28. Cross-reactions are possible between the O antigens of the morphologically similar *Proteus*, *Salmonella*, and *Escherichia* spp. So, serological grouping cannot be relied upon exclusively to distinguish among these genera.

19. Related Gram-Negative Bacilli

19-1. In addition to the organisms that make up the enterics, there is a fairly large group of Gram-negative, strictly aerobic, usually saprophytic bacilli in stool specimens. Many of these appear during the routine search for pathogenic enteric forms. Taxonomically, they fall within the following three families: Achromobacteraceae, Pseudomonadaceae, and Spirillaceae.

19-2. Organisms of the family Achromobacteraceae are grouped together because they attack glucose aerobically or not at all. We have already learned that the Enterobacteriaceae ferment glucose anaerobically as well as aerobically. The 7th edition of *Bergey's Manual* lists five different genera in the family Achromobacteraceae, but we will concern ourselves with only one, *Alcaligenes*. And although there are at least six different species in this genus, we will describe only *Alcaligenes fecalis* as being typical of the others. This microorganism is generally considered to be saprophytic, but on occasion it has been associated with various diseases of the blood, liver, lymph nodes, eyes, and kidneys, as well as enteritis. It is frequently isolated from the feces.

19-3. **Alcaligenes Fecalis.** This microbe does not ferment the usual carbohydrates, nor give positive reactions in the other conventional biochemical tests. Citrate is utilized as a source of energy, however. The organisms are generally motile. Microscopic and colonial morphology strongly resembles that of the *Salmonella*. The absence of carbohydrate fermentation on TSI agar (no color change in butt or slant) is a tipoff to the presence of *Alcaligenes fecalis*, although *Pseudomonas* can give the same reaction on TSI, citrate agar, and the motility test. The pattern of reactions shown in foldout 8 will assist in differentiation. Clear distinction among the morphologically similar *Alcaligenes, Pseudomonas, Herellea*, and *Mima* species can usually be found by inoculating the isolate to Sellers' agar to obtain the reactions presented in table 20.

like members of the Family *Pseudomonadaceae* that attack glucose aerobically and which usually produce a water-soluble pigment (blue, green, or brown; rarely rose, lilac, and yellow) are classified in the genus *Psudomonas*. It is the most important genus within the family and contains organisms clearly recognized as pathogens. This genus comprises 30-odd species of Gram-negative, strictly aerobic, usually saprophytic bacilli. For the most part they are found in the soil, water, or decomposing organic material. All species produce a water-soluble pigment.

19-5. Only two *Pseudomonas* species are generally considered to be pathogenic for man: *Psuedomonas aeruginosa* and *Pseudomonas pseudomallei*. Although the remainder are not infectious, they are important in food spoilage, e.g., milk and seafoods, when these products are stored at low temperatures. *Ps. pseudomallei* ("false glanders") causes a comparatively rare disease, melioidosis, in man. Found primarily in natural waters of India and the Southeast Asian countries, the bacillus has been isolated from the bloodstream and tissue lesions of man, rats, guinea pigs, rabbits, and certain of the larger domestic animals. It grows out on simple laboratory media, producing a yellow, creamy colony.

19-6. *Ps. aeruginosa* is often present in the normal intestinal tract, but in other parts of the body it can produce severe infections. Infections of the ear (otitis externa, otitis media) and mastoiditis are often due to this organism. It may infect surgical wounds and burns, causing the formation of a characteristic blue-green pus. Infection of the eyes and urinary tract also occurs. The organism has been responsible for peritoneal abscesses, septicemia, endocarditis, and meningitis. It sometimes invades the tissues of debilitated adults or infants. Antibiotic resistant strains are second only to the staphylococci in causing hospital infections.

19-7. *Pseudomonas aeruginosa* follows the fermentation pattern we saw with *Alcaligenes fecalis*. The identification steps charted in foldout 8 can be used for differentiation, and Sellers' agar reactions (table 20) are also helpful. Perhaps the most striking feature of *Ps. aeruginosa* is its ability to produce a blue or blue-green pigment. This pigment does not color the colony, but readily diffuses throughout the surrounding medium, as seen in foldout 4B, detail P. There are two pigments—one is soluble in chloroform, the other in water. In broth media a blue-green pigment is usually visible at the surface, but sometimes the pigment diffuses throughout the broth. If color is not visible, aerating the broth by gentle shaking will bring out the color. A sweet, aromatic odor can usually be detected in growing *Pseudomonas* cultures.

19-8. **Vibrio Cholerae.** The family *Spirillaceae* contains two genera of medical interest—the spiral forms in the genus *Spirillum* and the curved rods (comma shapes) of the genus *Vibrio*. *Spirillum minus*, the causative agent of one form of "rat bite fever," will be discussed with the morphologically similar spirochetes in a later chapter. The vibrios comprise about 34 species, of which only *Vibrio cholerae (V. comma)* holds clinical significance. It causes cholera in man.

19-9. The cholera vibrios remain confined to the intestinal tract, where they multiply rapidly and release an endotoxin upon lysis of the bacterial cells. The endotoxin is extremely irritating to the intestinal mucosa. This causes vomiting and profuse diarrhea (rice water stools), resulting in severe dehydration, anuria, hypochloremia, acidosis, and circulatory failure. The mortality rate ranges between 30 and 50 percent in un-

TABLE 21
CULTURAL CHARACTERISTICS OF VIVRIO CHOLERA

Test	Reaction
Motility	Positive
Cholera-red reaction	Positive
Carbohydrate fermentation (acid, no gas)	Glucose, galactose,maltose, sucrose, and mannitol-positive in 1 to 3 days. Lactose and dulcitol-negative
Hemolysis (tube method)	Nonhemolytic
Hydrogen sulfide production	Negative
Gelatin liquefaction	Positive 18 to 24 hours.

treated cases. Cholera is transmitted from infected individuals or convalescing carriers through contaminated food and water.

19-10. Stool specimens for *Vibrio cholerae* should be collected within the first 24 hours of the disease. Rectal swabs may be used if obtained during the acute phase of the illness. Swabs cannot be relied upon during the convalescent phase. Specimens collected during convalescense can be used if obtained by purging, although this does not be relied upon during the convalescent phase. Specimens collected during convalescense can be used if obtained by purging, although this does not yield 100 percent recovery of the vibrio. Fecal material, or preferably mucus flecks exhibiting vibrios in Gram stained smears, are picked with a wire loop and streaked on plates of blood agar, nutrient agar, and MacConkey's agar. In addition, particulate matter or a few drops of stool should be inoculated to a tube of 1.0 percent peptone water. Gohar broth may also be used to good advantage since the medium is inhibitory to Gram-negative organisms other than *V. cholerae.*

round, slightly convex, translucent, grayish-yellow, smooth, and granular in transmitted light. On blood agar the colonies are usually 1 to 2 mm. in diameter and exhibit a greenish zone. On MacConkey's agar the colonies are usually smaller and colorless. Growth of *V. cholerae* is more rapid (6 to 8 hours) in peptone water than is growth of other enteric bacilli. It is observed as a surface pellicle. Smears of the primary growth (colonies or pellicle) will reveal distinctly curved, Gram-negative rods with rounded ends. In most instances one end of a rod will appear more blunt than the other. This gives the organisms the shape of a "comma." The cells may be arranged singly, in S-shaped pairs, or rarely in short, spiraled chains. At this point it may be necessary to subculture the pellicle from peptone water to blood, nutrient, and MacConkey agars when growth is not found on the primary streak plates.

19-12. Table 21 shows some of the more important cultural characteristics of *V. cholerae*. In addition, the use of TSI and Lysine Iron agar is recommended to distinguish the vibrio from saprophytes giving similar cultural patterns. Table 22 provides an example of *V. cholerae* and *Aeromonas hydrophilia,* a harmless form.

19-13. The cholera-red reaction noted in table 21, if positive, is presumptive evidence of the cholera vibrio. Inoculate 1 percent trypotophan broth containing 0.001 percent potassium nitrate (final pH 7.8 to 8.0). After 24 hours incubation, add a few drops of commercial grade sulfuric acid. If indole is produced and nitrates are reduced, a deep rose color will develop rather quickly. This is the "cholera-red" reaction.

19-14. The hemolysin test is accomplished by mixing 0.2 ml. of a 5 percent saline suspension of washed sheep erythrocytes with an equal quantity of 24-hour broth culture of the vibrio under investigation. Incubate for 2 hours at 37 C., refrigerate overnight, and then examine for

TABLE 22
REACTION COMPARISONS OF TSI, KIA, AND LIA AGAR

		TSI	*KIA*	*LIA*
V. cholerae	Slant Butt H_2S	Alk Acid Neg	Alk Acid Neg	Alk Alk Neg
A. hydrophila	Slant Butt H_2S	Alk Acid Neg	Alk Acid Neg	Alk Acid Neg

hemolysis. *Vibrio cholerae* is nonhemolytic. Other vibrios are hemolytic. Although colonies of *V. cholerae* exhibit zones of greenish discoloration on blood agar, this is due to a hemadigestive process rather than to excretion of a true hemolysin.

19-15. Biochemical tests and morphological studies are usually supplemented by serological procedures to establish identification. Both polyvalent and specific antisera are available for slide agglutination tests. For epidemiological purposes these antisera as well as bacteriophages can be employed to pinpoint the strain or "type" of vibrio causing an outbreak of cholera.

CHAPTER 5

THE ACID-FAST BACILLI

The term "acid-fast" refers to a staining characteristic shown by certain microorganisms and not to a specific taxonomic designation. We learned in Volume 1 that bacilli whose cell walls contain large concentrations of fatty material resist penetration of the aniline dyes used in the Gram stain. To stain these organisms we must use special techniques (Ziehl-Neelsen, Kinyoun) to drive a dye into the cell wall by heat or chemical means. The dye cannot then be easily removed by conventional decolorizers containing strong acid. For that reason, the bacteria are said to be "acid-fast."

2. All of the acid-fast bacilli fall within the order actinomycetales, a group of Gram-positive rods that lie morphologically between the true bacteria and the filamentous fungi. Two families of the order are important from the clinical standpoint, but only three genera contain pathogens:

- Order V. Actinomycetales
  - Family I. Mycobacteriaceae
    - Genus I. Mycobacterium
  - Family II. Actinomycetaceae
    - Genus I. Nocardia
    - Genus II. Actinomyces

3. Most acid-fast species belong to the genus Mycobacterium, which claims such renowned pathogens as the tubercle bacilli and the causative agent of leprosy. This chapter will be devoted almost exclusively to the mycobacteria. In passing we need only to note one or two points with respect to genera of the family Actinomycetaceae. Members of the genus Nocardia are aerobic bacilli, some weakly acid-fast, that grow well on common laboratory media. The pathogenic species are N. farcinica and N. asteroides. The first is associated with a chronic tuberculosislike disease of cattle. N. asteroides has been isolated from absesses in man, and it also causes conditions resembling pulmonary tuberculosis.

4. There are two species of the anaerobic (but non-acid-fast) genus Actinomyces of general interest. A. bovis can be found in lesions of cattle and hogs, and the disease "lumpy jaw" draws its descriptive name from the type of swollen tissue produced by the infection. A. israelii, whose habitat is the human mouth and tonsillar crypts, has been implicated in tumorlike processes in various parts of the body. The pathogenic actinomycetes (Nocardia and Actinomyces) are recognized morphologically in the laboratory as Gram-positive, sometimes partially acid-fast rods with beaded and branching shapes. Rapid growth on ordinary culture media distinguishes them from the tubercle bacilli.

20. The Agents of Leprosy and Tuberculosis

20-1. Leprosy is an ancient disease that was well known as early as 1400 BC. It still occurs in certain Asiatic countries, in South America, and to a limited extent in the Southern United States.

2278

Mycobacterium leprae, also referred to as Hansen's bacillus, was discovered in 1878. The organism spreads mainly by personal contact, but exposure over a period of years seems to be necessary for infection to take place. Details of the actual mechanism of transmission are still lacking.

20-2. The acid-fast bacilli of leprosy are found in large numbers in the nasal secretions. The observation of typical M. leprae in the small mucoid particles of nasal secretions and in material aspirated from the lesions constitutes the major means of laboratory identification today. The organism has not been successfully cultivated on artificial media, but it can be propagated on the footpad of mice. Morphologically, Hansen's bacillus closely resembles the tubercle bacillus. The cells are slender rods measuring from 1.0 to 8.0 microns long and 0.3 to 0.5 microns wide. They are acid-fast and may appear beaded. The bacilli are generally found within tissue cells but may also be seen extracellularly. Characteristically, the bacterial cells are arranged in groups or packets, side by side.

20-3. Tuberculosis is also one of our oldest diseases. The infectious nature of the ailment was suspected during the 16th century, and Villemin showed in 1865 that tuberculosis can be transmitted from one person to another. But it was Robert Kock, the German physician, who first isolated and identified the causative agent in 1882.

20-4. Excluding the leprosy bacillus, we can place the 14 *Mycobacterium* spp. into four categories on the basis of cultural characteristics and biochemical tests. Members of all groups except the saprophytes cause tuberculosis or a clinically similar illness.

- *Mycobacterium tuberculosis.*
- The related species, *M. bovis, M. avium,* and *M. ulcerans.*
- The atypical mycobacteria.
- The saprophytes.

20-5. The human and bovine tubercle bacilli (*M. tuberculosis* and *M. bovis*) are the principal agents of tuberculosis in man. Human strains are almost always responsible for the classic pulmonary disease. Infections with the bovine species are occasionally pulmonary, but more often involve other tissues. Bovine tuberculosis is not common in adults, but it does occur with some frequency in children, especially in certain European countries. The avian tubercle bacillus (*M. avium*), found in wild birds and domesticated fowl, is reported from time to time as the cause of human infections.

20-6. Tubercle bacilli usually enter the body by way of the respiratory or alimentary tract. In man, the respiratory tract is the most frequent and important portal of entry. Infection arises through aerosol droplets and contaminated dust particles. Infections are also acquired from fomites (towels, drinking cups). The consumption of unpasteurized milk or inadequately cooked meat from infected cattle is an important source of infection where bovine tuberculosis is not well controlled.

20-7. Following initial infection, tubercle bacilli form primary and secondary lesions. The organisms may spread to various tissues via the lymphatic system, bloodstream, or by direct extension. Bloodstream dissemination can result in the so-called miliary, or "mustard seed," tuberculosis. Practically any tissue of the body is susceptible to invasion; however, more than 90 percent of the deaths from tuberculosis are due to the pulmonary form.

20-8. Our grouping of the mycobacteria listed *M. ulcerans* along with the bovine and avian forms in the second category because of a similarity in cultural properties that will be discussed later in the chapter. Although M. ulcerans is not ordinarily implicated in typical tuberculosis, the microorganism has been isolated from skin ulcers in man.

20-9. The third category, "atypical mycobacteria," refers to a number of anonymous acid-fast bacilli whose composite characteristics preven their being classified with any of the established species of *Mycobacterium.* Many of these forms produce a disease which is clinically similar to tuberculosis; yet, culturally, they exhibit several features which set them apart from the human, avian, and bovine species. These organisms have no clearly established animal hosts. It is not known precisely how they are transmitted from one individual to another, but some of them can cause a severe and often fatal disease in man.

20-10. The Runyon classification of the atypical acid-fast bacilli uses cultural and morphological properties, animal studies, and metabolic traits to assign these organisms to four subgroups. These subgroups are also given descriptive names:

Group I—Photochromogens
Group II—Scotochromogens
Group III—Nonphotochromogens
Group IV—The rapid growers

20-11. The photochromogens can produce pulmonary disease in man. The organisms form a yellow pigment in the presence of light; hence, the name photochromogen. *M. kansasii,* commonly called the "yellow bacillus," is the most frequent isolate from this group. Group II, scotochromogens, are exemplified by *M. scrofulaceum,* which produces a yellow or orange pigment whether cultivated in the light or in darkness. Not generally considered to be pathogenic, this organism has nevertheless been associated with cases of lymphadenitis in children.

20-12. The nonphotochromogens (Group III), often referred to as the "Battey bacilli," cause a form of tuberculosis clinically indistinguishable from the pulmonary infections brought on by *M. tuberculosis* and *M. kansasii.* Unlike the true tubercle bacillus, the Battey strains often show a primary drug resistance that makes treatment difficult. Group IV of the Runyon scheme contains the "rapid growers," so labeled because growth occurs in 3 to 4 days instead of the 2 to 3 weeks characteristic of the other mycobacteria. Most of the rapidly growing isolates from sputum, abscesses, and other clinical specimens will be *M. fortuitum.*

20-13. Going back to the major groupings set forth in paragraph 20-4 (not to be confused with

Runyon's subgroupings within the atypical acid-fast forms), we find the fourth category of mycobacteria to be the saprophytes or nondisease producers. The saprophytes *M. smegmatis* and *M. phlei* are often isolated from clinical specimens. Because these species are rapid growers they are sometimes classified with Group IV of Runyon's atypical acid-fast bacilli, but neither of the two has been implicated in disease. *M. smegmatis,* commonly referred to as the "smegma bacillus," is found occasionally in urine specimens. This bacillus is very difficult to distinguish microscopically from the pathogenic acid-fast bacilli. The organism is widely distributed in water, soil, and dust and can be isolated from human smegma. *M. phlei* is also found in the soil, in dust, and on plants. Commonly called the "timothy grass bacillus," the organism forms a dark-yellow, soft, waxy colony.

21. Distinguishing Characteristics of Tubercle Bacilli

21-1. As with most bacterial species, cellular size and shape of the mycobacteria differ with the environment in which the organisms are found—the culture tube and clinical specimen from various anatomical sources. Sputum, urine, and gastric washings are most often received in the laboratory; but tissue specimens, lymph aspirations, and pleural, pericardial, spinal, or joint fluids are submitted upon occasion. We learned in Chapter 4 of the first volume how to concentrate and digest urine, sputum, and gastric washings prior to microscopic examination and culturing. The other body fluids are not ordinarily digested, but the bacilli in them should be concentrated by centrifuging the specimen and using the sediment for making smears.

21-2. **Morphology.** Generally, we expect to see cells ranging in size from 0.3 to 0.6 micron in width and 0.6 to 4.0 microns in length, appearing either as slightly curved or straight rods. They may be found as short cells or as threadlike shapes. They sometimes appear branched or swollen, with a beaded effect. In tissues and exudates tubercle bacilli often take the form of small, thin rods with rounded ends. They may be straight or slightly curved. In smears from cultures, longer filamentous forms are occasionally observed, as well as swollen or club-shaped cells. Tubercle bacilli occur singly, in small groups, or frequently in clumps of indiscernible individual cells. Detail Q of foldout 4B illustrates a typical acid-fast smear. None of the mycobacteria produce spores. Virulent strains often have a capsular substance, especially when grown on a serum-enriched medium.

21-3. The result of microscopic examination of direct smears from clinical material is often reported as positive or negative for acid-fast bacilli. However, the National Tuberculosis Association suggests the following reporting format:

- Three to nine bacilli per *slide*—report as "rare."
- Ten or more cells per *slide*—report as "few."
- Ten or more in most oil immersion *fields*—report as "numerous."
- If less than three positive fields are found—prepare a new smear.

Some workers differentiate microscopically between typical acid-fast forms—i.e., the long, slender, sometimes beaded bacilli; and atypical organisms—the short, broad, pale-staining or branching cells. This distinction is difficult to make, at best, and should not be attempted without extensive laboratory experience in observing the tubercle bacilli.

21-4. Colony morphology among the mycobacteria ranges from the dry, nodular, rough, "cauliflower" growth typical of the human tubercle bacillus (seen in detail R of foldout 4B) to the smooth, moist, glistening slant of a scotochromogenic mycobacterium shown in detail S of foldout 4B. Note, however, in detail T of foldout 4B that the photochromogens of Runyon's atypical classification, as well as others, can look deceptively like the human strain seen in detail R of foldout 4B.

21-5. Most of the mycobacterial colonies are buff, off-white, or cream colored. Some of these nonpigmented strains give a hint of faint yellow or pink. The highly pigmented atypical bacilli of the Photochromogen and Scotochromogen groups appear bright yellow, orange, or brick red under certain cultural conditions. Two examples are given in details S and T of foldout 4B. The influence of light on pigment production is an important factor in identification, as we will emphasize later in the chapter.

21-6. **Cultural Properties.** The mycobacteria are aerobic and will not grow under strict anaerobic conditions. An incubation temperature of 37° C. is near the optimum. Growth is slow, usually requiring 2 weeks or longer, as we can judge from the data in table 23. The tendency of the human, avian, and bovine species to form colonies only at elevated temperatures (no growth at 25° C.) is significant in distinguishing them from the atypical groups.

21-7. We pointed out in Volume 1 that an enriched medium such as Lowenstein-Jensen's or Petragnani's is required for growth, and even

TABLE 23
GROWTH-TEMPERATURE RELATIONSHIP

ORGANISM	COLONY FORMATION (DAYS)	
M. tuberculosis	No growth	12 to 25
M. bovis	No growth	25 to 40
M. avium	Slight or no growth	21 to 28
M. ulcerans	60	60
Photochromogens (Group I)	12 to 25	10 to 21
Scotochromogens (Group II)	12 to 25	10 to 21
Nonphotochromogens (Group III)	12 to 25	10 to 21
Rapid Growers (Group IV)	7	3 to 5

with these enriched media the cultures should be kept for at least 8 weeks before discarding as negative. Remember to examine the tubes every 3 or 4 days, and periodically loosen the screw caps of the culture tubes to replenish the oxygen supply. Media for the mycobacteria are usually purchased pretubed because of the labor and expense of preparation. The principal growth promoting constituents are homogenized whole egg, glycerol, asparagine, and potato starch. Malachite green is added to inhibit the growth of contaminating organisms.

21-8. Recently, Middlebrook 7H10 agar with oleic acid, dextrose, and citrate enrichment has been added to the arsenal of diagnostic media. It is excellent for primary isolation and susceptibility testing of *M. tuberculosis.* This medium has several advantages. Early detection of colonies with the aid of the microscope is possible because the medium is clear. It permits easy separation of mixed colony types, and contamination with other bacteria or fungi does not alter or destroy the agar base medium as quickly as the egg base medium of Lowenstein-Jensen or Petragnani's. Whereas up to 8 weeks' cultivation is required on egg base media, Middlebrook 7H10 shows growth within 3 weeks. Middlebrook 7H10 must be relatively fresh, however, and cul-

TABLE 24
PIGMENT PRODUCTION BY ATYPICAL ACID-FAST BACILLI

Group	Name	Pigment when grown in light	Pigment when grown in dark
I	Photochromogens	+	-
II	Scotochromogens	+	+
III	Nonphotochromogens	(-)	(-)
IV	Rapid growers	(+)	(-)

KEY:

+ = Reaction, growth or production
- = Nonreactive or no change
() = Usual reaction

NOTE:

Atypical acid-fast bacilli grow at 22° to 37° C. Pigments are usually yellow to orange.

TABLE 25
BIOCHEMICAL PROPERTIES OF THE MYCOBACTERIA

Organism	*Niacin Production*	*Catalase*		*Sulfatase*
		Room Temperature)	(68°C.)	
M. tuberculosis	+	+	-	-
M. avium	-	+	+	-
M. bovis	±	+	-	-
M. ulcerans	-	+	+	
Photochromogens	-	+	+	+
Scotochromogens	-	+	+	+
Nonphotochromogens	-	+	+	+
Rapid growers	-	+	+	+

tures must be incubated under increased CO_2 tension. It is important to note that even with egg base media the presence of CO_2 definitely stimulates the rate of growth of the tubercle bacilli.

21-9. From table 24 it is apparent that the formation of pigment as a function of light intensity proves useful in identification. We stated earlier that the human, avian, and bovine species form little or no true pigment, whether in light or darkness. But in the so-called atypical species of the Runyon groups pigment changes take place. Colonies of Group I, the photochromogens, are yellow to brick-red when grown in the light, but they lack pigment in the absence of light. Colonies that have grown for about a week can be made to develop pigment in 6 to 12 hours if they are exposed to a 30-watt lamp at a distance of 14 to 15 inches. (Mature colonies will not usually react.) In fact, a yellow pigment is produced within 24 hours after only 1 hour's exposure to bright light. The pronounced color change is shown in detail T of foldout 4B.

21-10. The scotochromogens (Group II) elaborate a yellow or orange pigment whether grown in light or darkness. The Group IV rapid growers resemble the photochromogens in response to light, but the rate of colony development easily differentiates these groups. The nonphotochromogens (Group III) are distinctive among the atypical species in producing little or no pigment in their colonies.

22. Special Diagnostic Tests

22-1. The identification of *Mycobacterium* isolates rests partly on the comparison of several characteristics which we have already covered: the temperature range for growth; the period required for colony formation; and the effect of bright light on pigment production. In addition, there are certain biochemical procedures that are useful in differentiating the various species and groups. The results shown in table 25 are subject to considerable variation among strains of a given species, however. As a further aid to identification, laboratories equipped to perform animal studies can confirm the pathogenicity of isolates by inoculating guinea pigs, rabbits, and mice.

22-2. **Biochemical Procedures.** As we find in table 25, the human strains of mycobacteria, whether virulent or not, are able to form *niacin* (nicotinic acid) when grown on one of the egg media we have discussed. Bovine species are usually negative, and avian species are consistently negative. Niacin in the culture is detected by a color reaction with cyanogen bromide and aniline.

22-3. To test for niacin production we pipette 1.0 ml. of sterile saline to a 3-week, or older, culture slant. (Be sure that there is no niacin-containing additive such as penicillin in the medium.) In order to extract the niacin, puncture the slant around the growth with the dropper pipette tip. The colonies should be covered with the saline and allowed to stand for about 30 minutes. Transfer a portion of the saline to a small screw-capped test tube. Add an equal portion of aniline and cyanogen bromide solution to the saline extract. If niacin is present a yellow color will appear almost instantly. A positive and a negative niacin test are shown in detail U of foldout 4B. CAUTION: cyanogen bromide is a tear gas, so perform the test in a well-ventilated area or a fume hood.

22-4. All acid-fast bacilli produce catalase, but the production of this enzyme decreases when the bacilli become resistant to the therapeutic agent, isoniazid. The slackening of catalase activity is correlated with a weakening of virulence for the guinea pig. It is also possible to subgroup acid-fast bacilli on the basis of their catalase activity at different temperatures and pH. At 68° C. and pH 7.0 the catalase of human and bovine tubercle bacilli is selectively inactivated. Under the same conditions all other acid-fast species are catalase-positive.

22-5. To check the catalase activity at room temperature prepare a 1:1 mixture of 10 percent tween 80 and 30 percent hydrogen peroxide. Add 0.5 ml. of the mixture to the Lowenstein-Jensen slant. Bubbling indicates a positive catalase test. To test catalase activity at 68° C. add several loopfuls of mycobacteria from a slant to 0.5 ml. of a phosphate buffer solution (pH 7.0) in a test tube. Incubate at 68° C. in a water bath for 30 minutes. Add 0.5 ml. of the tween-hydrogen peroxide mixture to the buffer-growth solution and observe for the bubbling indicative of a positive reaction.

22-6. The sulfatase test recorded in table 25 measures the ability of mycobacteria to form an enzyme, sulfatase, when grown in a medium containing 0.001 molar tripotassium phenolphthalein disulfate. The enzyme, if present, liberates phenolphthalein, which is detected by adding an alkali to develop a characteristic red color.

22-7. The test is performed by inoculating a portion of an actively growing Lowenstein-Jensen culture to Wayne's phenolphthalein sulfatase agar by surface streaking and stabbing. We incubate for 72 hours at 37° C., then add 0.5 ml. of 1*M* sodium carbonate solution to the inoculated medium and to an uninoculated control medium. A positive reaction is observed as a pink color formed in the agar or the supernatant fluid. Note in table 25 that the atypical species are sulfatase-positive and the avian, bovine, and human species are negative.

22-8. **Sensitivity Testing.** We learned in Chapter 5 of Volume 1 how to set up sensitivity tests to measure the antimicrobial activity of antibiotics. The resistance or susceptibility of the tubercle bacilli to drugs can be assessed by applying the same principles. The procedure for preparing the inoculum and the parameters for choosing the direct or indirect technique are sufficiently unique with the acid-fast bacteria, however, as to merit a brief but separate discussion.

22-9. The selection of a method, i.e., direct or indirect, depends on the number of bacilli seen on stained slides prepared from the digested specimen. If the smear shows only an occasional acid-fast cell or is negative, drug-free media should be inoculated to obtain sufficient growth for *indirect* testing. (The organisms are cultured before seeding to antibiotic test media.) Rarely can an accurate *direct* determination of susceptibility be accomplished when less than 10 organisms are found on an entire smear (direct inoculation of antibiotic test media with the specimen). The *direct* test on either *tube* or *plate* is performed on a saline concentrate of the sediment obtained through digestion of the specimen Dilution is made according to the number of organisms seen on the smear under oil immersion.

22-10. If there is less than one cell per field, two plates are inoculated in the direct *plate* technique, one from undiluted sediment and the other from a 1:10 dilution in saline. If 1 to 10 bacilli are seen per oil immersion field, dilutions of 1:10 and 1:20 are inoculated. If more than 10 are found, dilution factors of 1:20 and 1:30 are used. The culture plates are prepared from petri dishes of Middlebrook 7H10 agar with four sections. Three of the quadrants contain media with a different antimicrobial agent such as isoniazid (INH), para-aminosalicylic acid (PAS), and streptomycin. The fourth quadrant serves as a control. Plates are inoculated with 3 drops of the sediment or diluted specimen to each quadrant and then placed in an upright position at 35° C. to 37° C. for 3 weeks, preferably in an atmosphere of 2 to 5 percent CO_2. Growth is reported according to some numerical system such as the following:

- Confluent growth (over 500 colonies)—report 4+.
- From 200 to 500 colonies—report 3+.
- From 100 to 200 colonies—report 2+.
- From 50 to 100 colonies—report as 1+.
- Report the number of colonies if fewer than 50 colonies appear in the quadrant.

22-11. The direct *tube* method uses a Lowenstein-Jensen medium containing antimicrobial agents such as isoniazid, para-aminosalicylic acid, streptomycin, viomycin, cycloserine, pyrazinamide, ethionamide, or others. These tubes are available commercially. If the smear of the sediment shows numerous acid-fast bacilli, inoculate 0.1 ml. of the undiluted sediment to the control tube (no antimicrobial agent). The entire surface of the slant should be covered. This can be accomplished using sterile pipettes or cotton-tipped swabs. The remaining sediment should be diluted according to the number of bacilli seen on the smear, using a diluent such as a 0.1 percent albumin phosphate buffer, pH 6.8.

22-12. Inoculate two control tubes and one tube each of the drug-containing media. Incubate them at 37° C. with the tubes placed in a horizontal position for the first 7 days. After 4 weeks of incubation, report the extent of growth on the control tube (which should show a 3+ or 4+ growth) as well as the growth on each of the drug-containing tubes.

- Report confluent growth as 4+.
- Heavy growth, not confluent, is reported as 3+.
- More than 200 discrete colonies are reported as 2+.
- Report 50 to 200 colonies as 1+.
- If there are fewer than 50 colonies, report the actual number.

22-13. Whenever possible, the direct method (plate or tube) for testing drug susceptibility should be used. But, if the smears reveal too few organisms for direct seeding, then the indirect method applies. As with the direct techniques, we can use either plates or tubes. Emulsify at least five colonies of the organism from a pure culture in 3 ml. of tween-albumin broth (TAB) with the aid of several sterile glass beads. To prevent splattering, use a screw-capped tube. Place the tube in a test tube mixer for 5 to 10 minutes; then allow the larger particles to settle. Adjust the supernatant to match MacFarland #1 standard with sterile distilled water or 0.85 percent saline. (The MacFarland #1 standard can be prepared by adding 0.1 ml. of 1 percent barium chloride solution to 9.9 ml. of 1 percent sulfuric acid.) After the supernatant has been adjusted, make 1:20 and 1:40 dilutions to inoculate the quadrant plates as in the direct method. Alternatively, we can inoculate the surface of tubed antibiotic test media with 0.1 ml. of the supernatant fluid. Incubate and report the growth as outlined for the direct tube method.

The Spirochetes

TRADITIONALLY, THE term "spirochete" embraces a host of slender, flexible, filamentous, corkscrewlike organisms distributed widely in water, soil, plants, animals, and man. Most of these bacteria are saprophytes, but a few are pathogenic. Those pathogenic for man cause such diseases as yaws, relapsing fever, syphilis, and certain hemorrhagic disorders.

2. Spirochetes were among the earliest microorganisms to be seen. Van Leeuwenhoek, soon after his invention of the light microscope, saw and described various types of spirochetes from his own mouth and gums. The first spirochete associated with a specific disease was *Borrelia recurrentis*, discovered in the blood of a patient with relapsing fever around 1873. The causative agent of syphilis, *Treponema pallidum*, was not described until about 1905, although the disease had been recognized clinically since the time of Columbus' voyage to America.

3. The true spirochetes belong to the order Spirochaetales, whose subordinate taxonomic groups are shown in the following classification scheme from *Bergey's Manual:*

Order IX. Spriochaetales
  Family I. Spirochaetaceae
    Genus I. *Spirochaeta*
    Genus II. *Saprospira*
    Genus III. *Chistispira*
  Family II. Treponemataceae
    Genus I. *Borrelia*
    Genus II. *Treponema*
    Genus III. *Leptospira*

4. By definition, members of the order take the form of slender spirals. They range from 6 to 500 microns in length, and they possess at least one complete turn in the spiral. All species are motile. The order can be subdivided into two families on the basis of cell length: Those organisms 30 to 500 microns in length and with a definite protoplasmic structure are placed in the family Spirochaetaceae. Those with a length of 3 or 4 to 20 microns or so, and without protoplasmic structure, are placed in the family Treponemataceae.

5. The free-living spirochetes of the family Spirochaetaceae can be disregarded as the cause of disease in humans. This chapter will, therefore, be concerned mainly with the family Treponemataceae, each of whose three genera contains pathogens. In figure 19 we have sketched the typical morphology of each genus. Note that spiral length, the number of spirals, and to a lesser extent the amplitude of each spiral are characteristic.

6. Morphology is one of the three distinguishing properties—the other two are staining characteristics and oxygen requirements. That is, *Borrelia* are easily stained with aniline dyes, and this feature separates them from the *Treponema* and *Leptospira* which stain with difficulty. Differentiation of the latter two genera is based on oxygen requirements: *Treponema* are anaerobic, *Leptospira* are aerobic. Since the pathogenic spirochetes are not routinely cultivated on artificial media, however, microscopic morphology coupled with the clinical picture, augmented by serological tests in certain instances, is the principal means of distinguishing among the three genera.

23. The Genus Borrelia

23-1. There are 28 different species of *Borrelia*, many of them morphologically similar, but we will discuss only two from the laboratory standpoint: *Borrelia recurrentis*, which causes relapsing fever; and *Borrelia vincentii*, which causes ulcerative stomatitis (Vincent's Angina) and ulcerative lesions of the genitalia. In nature, a dozen species other than *B. recurrentis* give rise to a type of relapsing fever, but the laboratory procedures described in this chapter for identifying *B. recurrentis* are equally applicable to the others.

23-2. Relapsing fever is an ancient disease. An epidemic on the island of Thasos some 20 centuries ago was described by Hippocrates. Al-

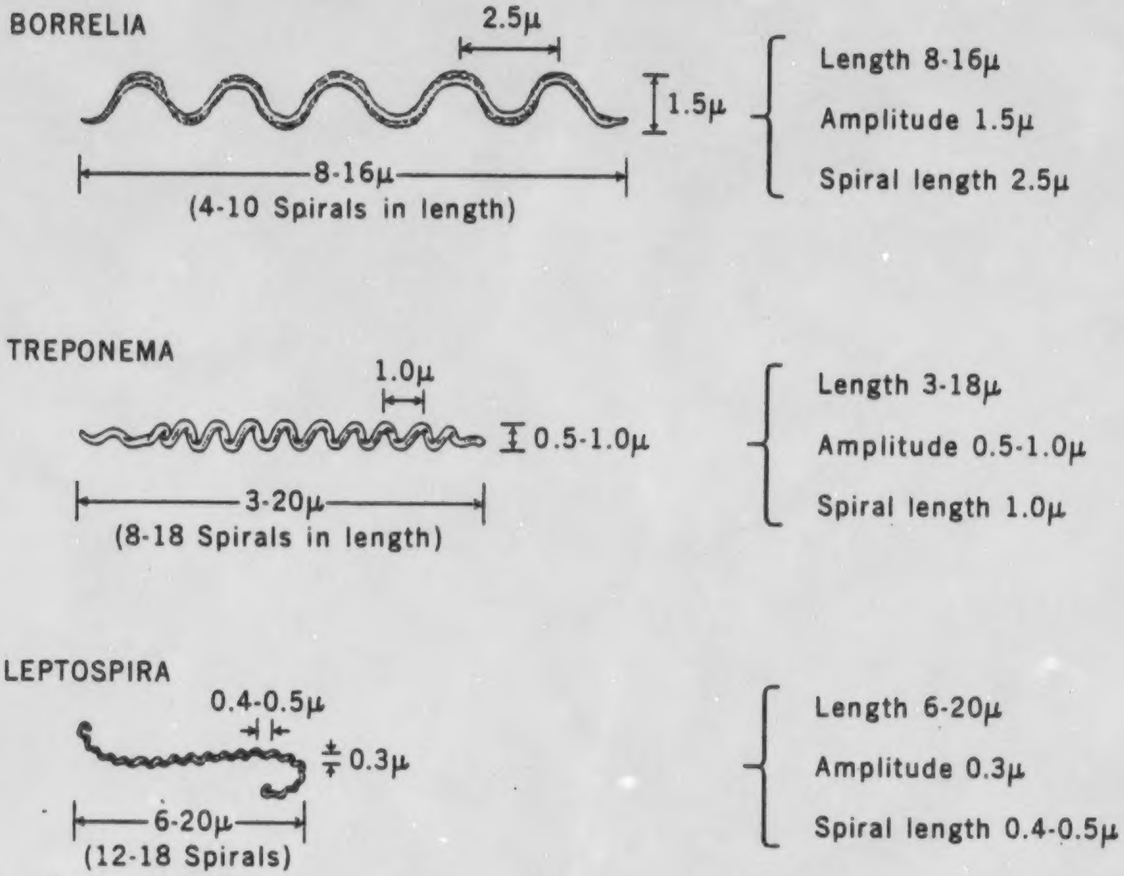

BORRELIA
2.5μ
1.5μ
8-16μ
(4-10 Spirals in length)
Length 8-16μ
Amplitude 1.5μ
Spiral length 2.5μ
TREPONEMA
1.0μ
0.5-1.0μ
3-20μ
(8-18 Spirals in length)
Length 3-18μ
Amplitude 0.5-1.0μ
Spiral length 1.0μ
LEPTOSPIRA
0.4-0.5μ
0.3μ
6-20μ
(12-18 Spirals)
Length 6-20μ
Amplitude 0.3μ
Spiral length 0.4-0.5μ

Figure 19. Major shapes of spirochetes.

though the disease is still prevalent in Central America, India, Africa, and parts of Europe, it has not been observed in epidemic form in the United States. The organism is transmitted from man to man by the body louse and from rodent to man by ticks.

23-3. A high fever develops from 3 to 10 days after the person has become infected. In the early stages there are numerous organisms in the bloodstream and occasionally in the urine. After 4 or 5 days, the fever subsides and the number of organisms in the bloodstream decreases. Those that remain take on rather atypical and bizarre forms and lose their motility. This period of low grade fever or normal temperature may last for 1 to 4 weeks before giving way to another febrile attack. Organisms may invade the spleen, liver, gastrointestinal tract, and kidney.

23-4. Morphologically, this spirochete is a spiral thread measuring 0.2 to 0.5 micron in width and 8 to 16 microns in length. It may possess from four to ten spirals, with most cells

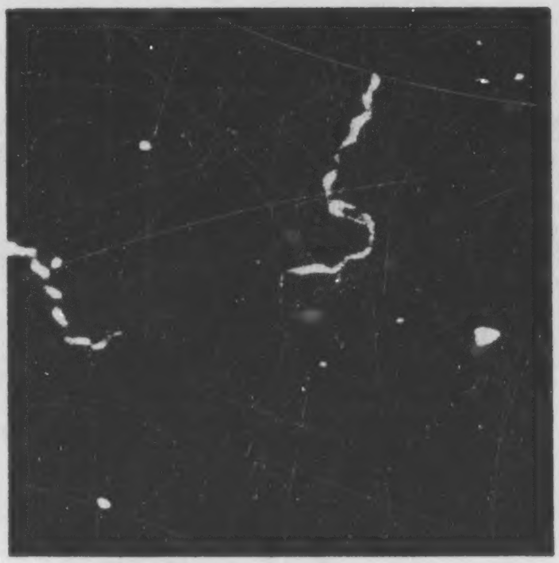


Figure 20. Photomicrograph of *Borrelia* under darkfield.

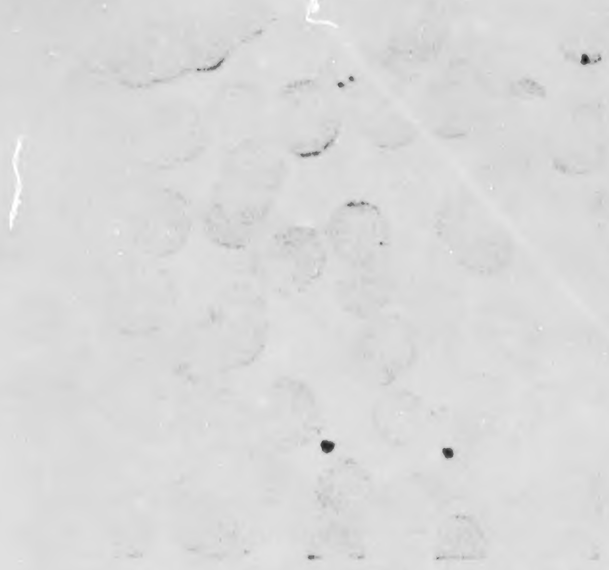


Figure 21. Photomicrograph of *Borrelia* in rat blood.

showing around five. Spirals are large and wavy with a distance of approximately 2.5 microns between them. Amplitude is about 1.5 microns as we have shown in figure 19. Occasionally, axial terminal filaments are seen, and these may give the appearance of flagella. The spirochetes can be stained with the Gram stain, in which they appear Gram-negative. The Romanowsky modification or Geimsa stain which stains the organism purple can also be used. The mechanics of staining cause some distortion and straightening of the spirals, and experience must be gained if this manner of generic identification is to be relied upon.

23-5. For best results in viewing morphology, a direct wet preparation is used. See, for example, the darkfield micrograph in figure 20. Under these conditions the actively motile spirochetes have a corkscrew movement along with lateral oscillation (side to side). The motion is not necessarily directional, and the organism may be seen to move back and forth.

23-6. The spirochetes of *Borrelia* can be artificially cultivated in liquid media such as ascitic fluid containing a small amount of rabbit kidney and several drops of citrated blood, final pH 7.2 to 7.4. Laboratory diagnosis is based, however, on finding the spirochetes in the blood during a period of fever. In a freshly collected specimen the organisms are actively motile under darkfield illumination. Since identification in the direct preparation is often difficult, a stained blood smear provides the opportunity for further study. Figure 21 permits a size comparison between the spirochete and surrounding red cells.

23-7. *Borrelia vincentii*, in association with the fusiform bacilli of the bacteroides group, causes serious infections of the mouth and throat. One of the best known diseases of this nature is Vincent's angina, commonly referred to as "trench mouth." Milder infections include stomatitis and gingivitis. Although infections are usually limited to the mouth, fuso-spirochetal symbiants may become primary or secondary invaders of the lungs, intestinal tract, skin, and genital area. It is possible for any organ of the body, including the brain, to become infected through metastases.

23-8. *Borrelia vincentii* is motile with a rapid vibratory motion which allows the spirochete to progress through the medium. Individual cells are approximately 7.0 to 12.0 microns in length with three to eight spirals. The organism stains Gram-negative. The anaerobe is hard to isolate and keep in pure culture. If cultivated in symbiosis (with other bacteria) in a meat infusion-ascitic fluid broth from which atmospheric oxygen is excluded, growth occurs fairly well.

23-9. To identify *Borrelia vincentii* as the causative agent of Vincent's angina, it is necessary to find the spirochete. In positive cases you will also find fusiform bacilli *(Fusobacterium fusiformis)* on the same stained slide. The Gram-negative fusiform bacilli appear as long or short, slender, curved rods with pointed ends. Occasionally they appear as long filaments. They occur singly or in pairs attached end to end as a result of incomplete fission. Size varies from 0.30 to 0.9 micron in width and 3 to 12 microns in length.

23-10. *Borrelia vincentii* can be stained by Loeffler's methylene blue, carbol-fuchsin, Giemsa's stain, Wright's stain, or the Gram stain. Smears from the deeper parts of the throat lesions or buccal ulcer most often show the spirochetes. The combination of fusiform bacilli and spirochetes seen on a Gram stained smear made from typical lesions is good evidence that *Borrelia vincentii* is the causative agent. There are many spirochetes which exist as part of the normal flora of the mouth, however. Spirochetes such as *Treponema microdentium* and *Treponema mucosum* can be mistakenly identified as *Borrelia vincentii*. Therefore, the association of fusiform bacilli with the spirochetes is an important element in correct identification.

24. The Genus Treponema

24-1. *Bergey's* 7th edition lists 8 species of the genus *Treponema*. Of these, *Treponema pallidum* causes the veneral disease syphilis in man; *Treponema pertenue* causes yaws, an ulcerative skin disease of the tropics. *Treponema microdentium* is found normally in the mouth, and

8

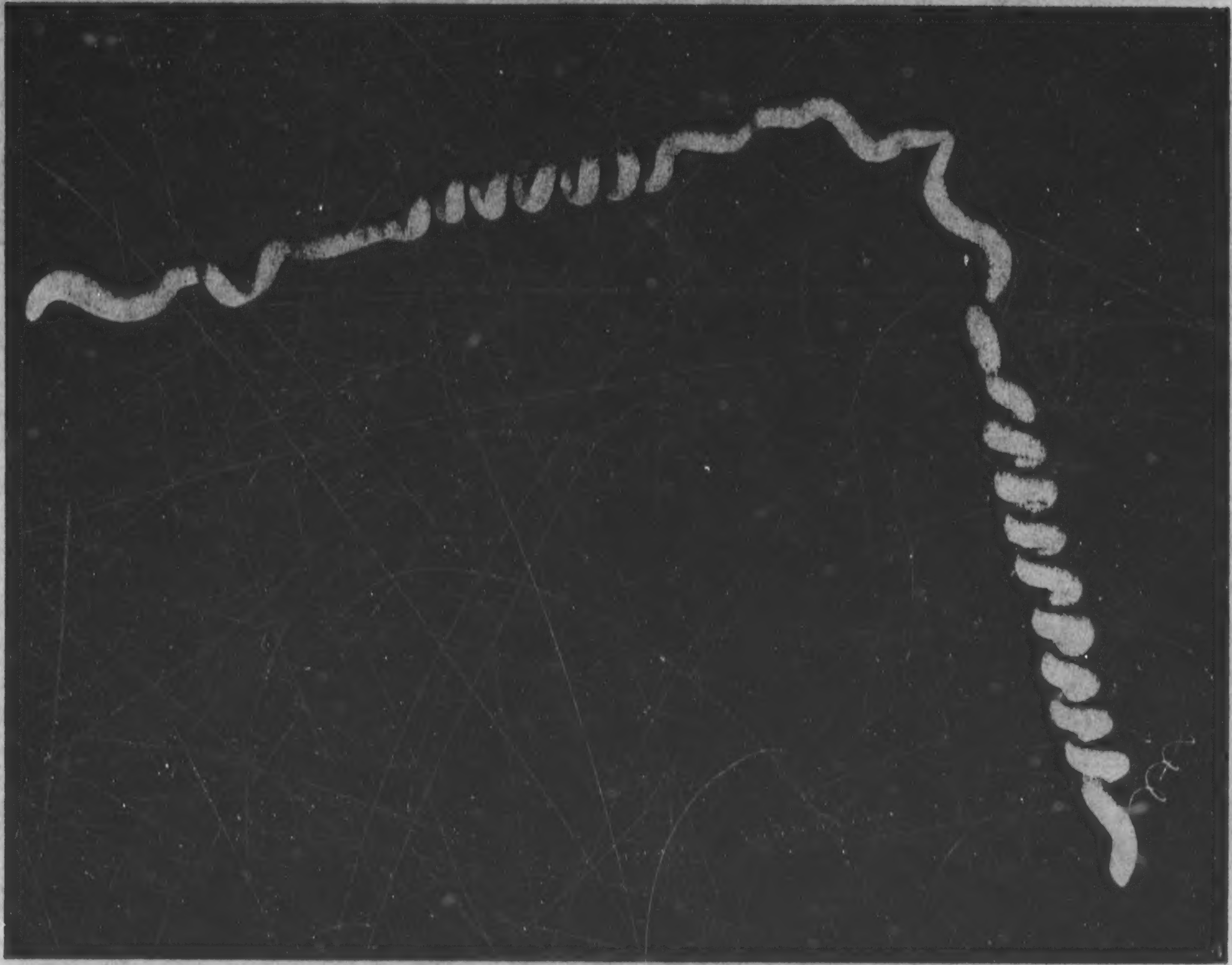


Figure 22. Photomicrograph of *T. pallidum* (two organisms).

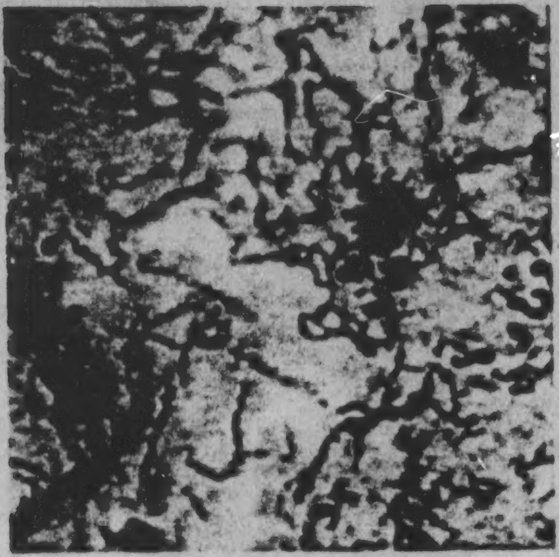


Figure 23. Stained smear of *T. pallidum* in tissue exudate.

Treponema mucosum is associated with the gum disease, pyorrhea alveolaris. *Treponema genitalis* has been implicated in veneral disease, but without definite proof.

24-2. *Treponema pallidum* (pale thread) is the etiologic agent of syphilis, a highly contagious disease transmitted from person to person through intimate contact, usually sexual. Infections may be transmitted occasionally through indirect means such as fomites (drinking cups, towels), but only when the time interval between contamination of the article and its subsequent contact with an individual is very short. For this reason, fomites used by persons with open, infective lesions should be considered highly contagious.

24-3. *Treponema pallidum* enters the skin or mucous membranes through minute breaks. The spirochetes remain for a time at the site of infection and multiply. The characteristic inflammatory response known as the chancre follows. This stage is referred to as primary syphilis, and the chancre forms within 10 to 90 days after infection. The chancre lasts from 1 to 3 weeks and may heal spontaneously. Primary syphilis is generally diagnosed by darkfield microscopic detection of *Treponema pallidum*, although a positive serologic test for syphilis will occasionally be obtained in this stage of the disease.

24-4. Within 2 to 12 weeks after the primary chancre heals, a generalized skin rash usually appears. This stage of the disease is referred to as secondary syphilis. Symptoms usually disappear within about 3 weeks and, in untreated cases, may reappear one or more times as relapses. In secondary syphilis the characteristic skin lesions contain *Treponema pallidum*. An increasingly positive serologic test is the rule. The subsequent latent stage of syphilis is usually shown by a persistently positive reaction in serologic tests. Late symptomatic syphilis, which can be expected to follow the latent period in untreated cases, can also be detected by reactive serologic tests.

24-5. The spirochetes of *Treponema pallidum* occur as very fine, delicate spiral forms having a flexible cylindrical body measuring approximately 0.25 micron in diameter and 5 to 16 microns in length. The spiral height (amplitude) is approximately 1.0 micron. The organism possesses from 8 to 15 regular spirals. Figure 22 is a photomicrograph of two spirochetes of *T. pallidum*. Motility is generally slow, although the cell rotates rapidly on its axis. During the rotational process, the organism bends to form circular as well as S shapes. In the material aspirated from the lesion of early syphilis, the cell tends to be elongated (like an overstretched spring) and movement is more snakelike. This is due to the greater density of the fluid caused by mucus present in the lesion.

24-6. Staining of *Treponema pallidum* by the Gram technique is usually unsuccessful. Although the organism may take the dye, the amount of protoplasm available for staining is so small that the necessary visual contrast is lacking. Staining can be accomplished satisfactorily, however, by using special techniques such as the silver impregnation method of Levaditi, or one of its several modifications. This technique is based on the coating of the surface of the treponeme with metallic silver to increase contrast. In the Fontana-Tribondeau method the cell surface is coated with reduced silver nitrate. In figure 23 we see stained spirochetes typical of *T. pallidum*. In the absence of silver impregnation materials, the nigrosin or India ink preparations can be used. These dyes do not stain the treponeme, but instead provide a black background in which the organisms stand out in relief. Recently, fluorescent antibody techniques have been used with some success.

24-7. The pathogenic treponemes have not been cultivated in the laboratory. An exception is the nonvirulent Reiter strain of *Treponema pallidum* that has been grown under certain conditions in thioglycollate or other anaerobic broths containing serum. Attempts to culture the treponemes in tissue or fertile eggs have failed. Laboratory diagnosis must therefore be based on staining, fluorescent antibody techniques, serological reactions, and the darkfield examination.

24-8. Let's take a look at the darkfield tech-

239

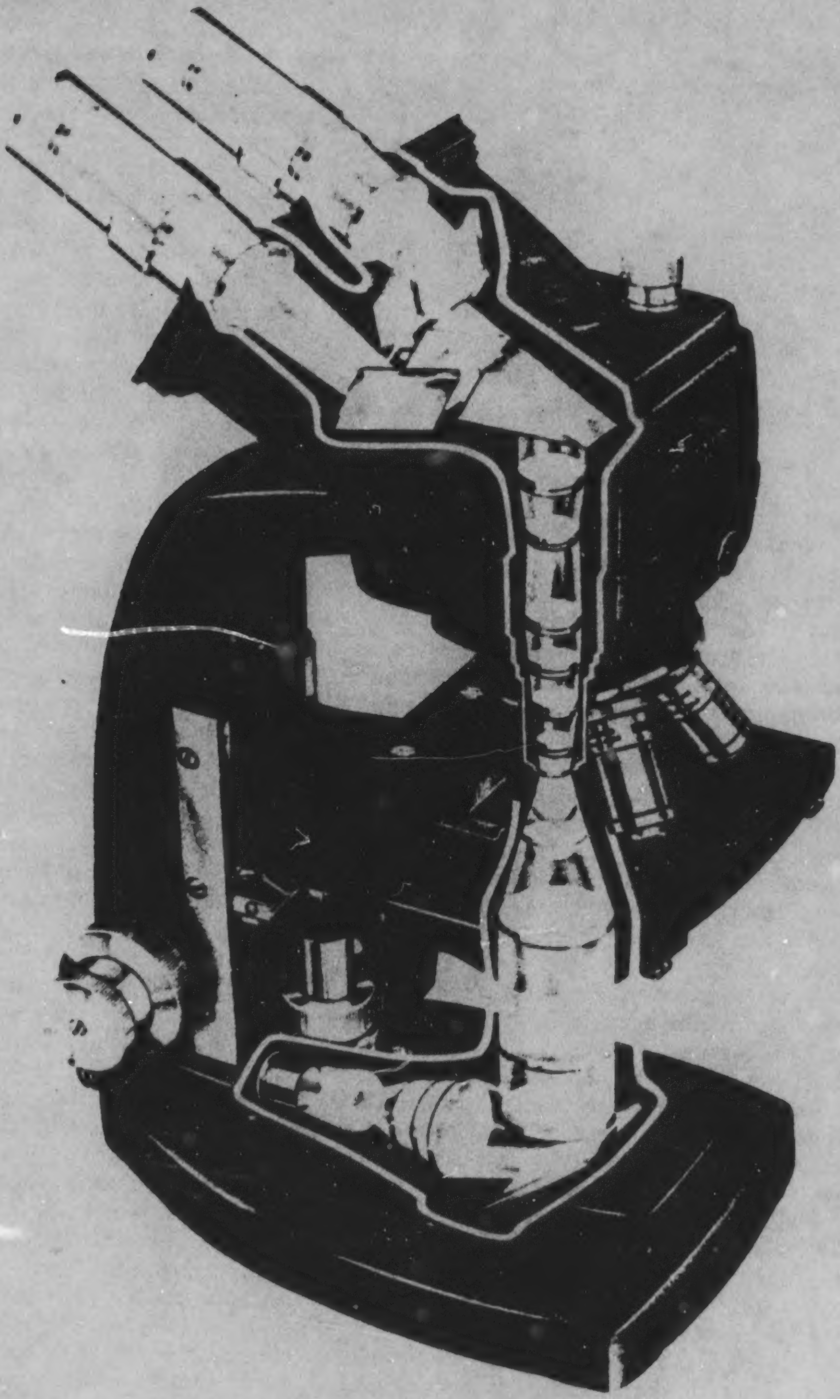


Figure 24. Binocular microscope—cutaway view.
(Courtesy Bausch & Lomb, Rochester, New York)

259

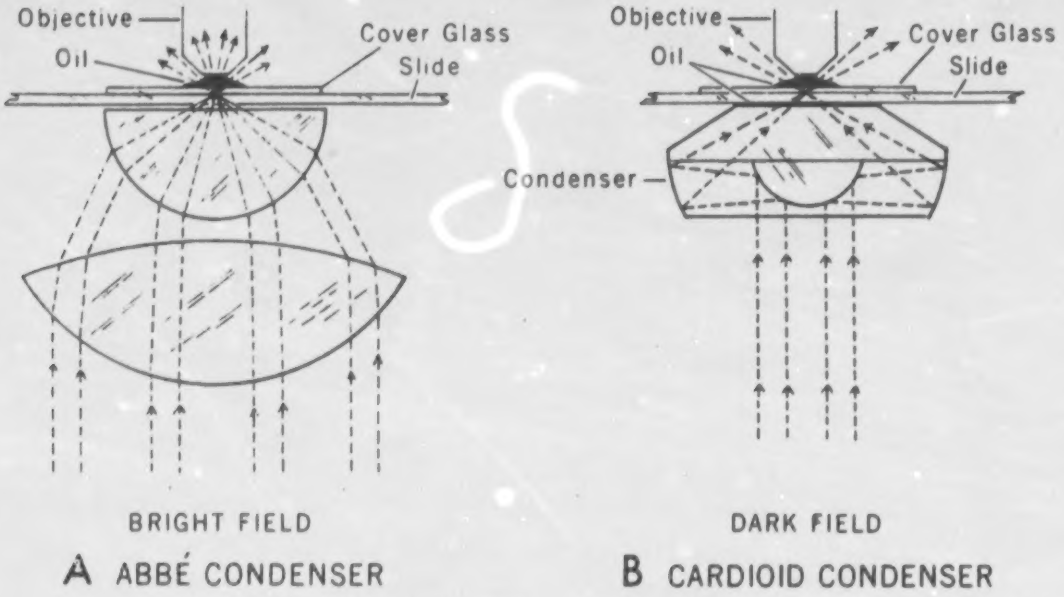


Figure 25. Brightfield versus darkfield condenser system.

nique that is employed during first examination of the syphilitic lesion. Most of the microscopy you have done has been with "brightfield" illumination. This form of microscopy was covered in detail in CDC 90411, Chemistry and Urinalysis. A cutaway of a conventional binocular microscope showing the light path is illustrated in figure 24. The inset is a closer view of the condenser area. In the darkfield setup, we replace the standard microscope condenser with a special darkfield condenser to provide oblique rather than direct lighting of the specimen.

24-9. In brightfield microscopy, the substage condenser provides a solid cone of light that is concentrated on the specimen, as we see in figure 25. In "darkfield illumination," the cone of light is hollow, leaving a dark central area. It is this darkened center that we see when we look through the microscope. Thus, the darkfield condenser provides scattered light that reflects off any object in the darkened field, giving us bright objects against a black background. In most laboratories that perform the darkfield examination, one microscope adapted for this procedure with special condenser and objective lenses is set aside for the purpose.

24-10. Collecting material from a syphilitic lesion for darkfield study must be done carefully. First, you must clean the lesion of surface crust, detritus and pus, with gauze or a cotton applicator. If the patient has treated his lesion with a germicidal agent, examination is deferred until all germicide has been removed by washing and several hours have elapsed. Primary lesions are then mildly traumatized to provoke a serum exudate. We do this by gently rolling the lesion between the gloved finger and thumb or by rubbing its surface with a dry cotton applicator. Hemorrhage should be avoided, although a few erythrocytes or pus cells are desirable to aid in obtaining a proper focus. Secondary lesions are merely cleansed with a saline gauze and abraded.

24-11. Fresh preparations for microscopic examination are made from accessible lesions by touching the slide to tissue juice and immediately placing the cover glass over the drop of fluid. Vaseline placed around the edge of the cover slip will prevent drying. If the lesion is less accessible, the fluid may be collected in a capillary pipette and placed on the slide. Examine immediately with the darkfield microscope for the

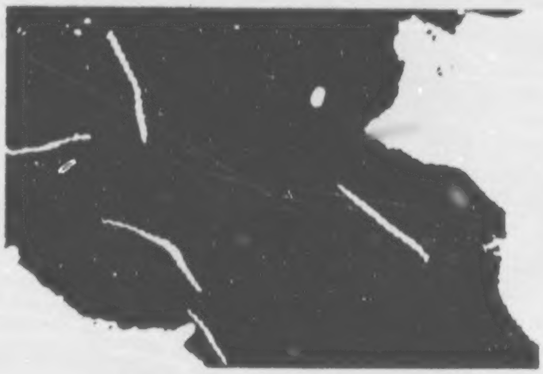


Figure 26. Darkfield of *T. pallidum*.

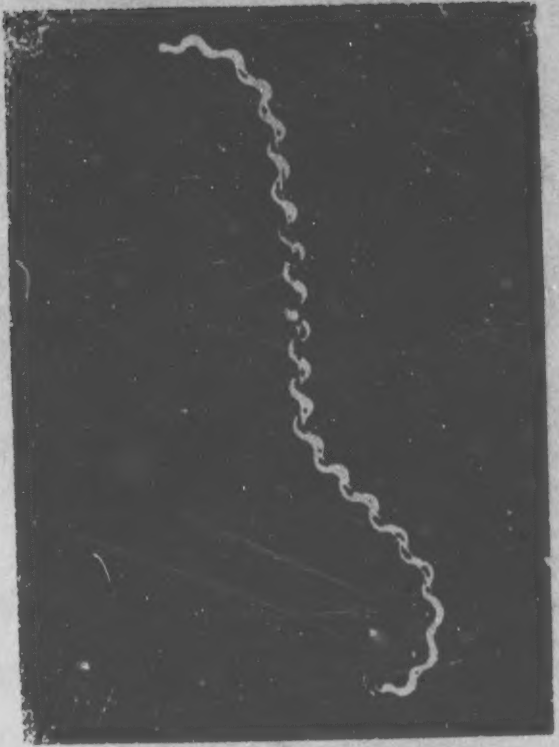


Figure 27. Electron photo of *Leptospira*.

characteristic morphology and motility of *T. pallidum*, seen in figure 26.

24-12. There are many saprophytic spirochetes that are not easily distinguished from *T. pallidum*. A diagnosis of syphilis based solely on the darkfield examination, especially of material from the oral cavity, is not valid because of the variety of saprophytic spirochetes present. Moreover, "artifact spirochetes" are sometimes mistaken for *T. pallidum* by those unfamiliar with the appearance of blood, pus, tissue cells, and bacteria under darkfield illumination. Wavy, filamentous structures may actually simulate spirochetes. The physician uses the result of this microscopic examination as only one factor in arriving at a correct diagnosis.

24-13. When facilities for darkfield microscopy are not locally available, tissue fluids may be forwarded to some other laboratory for study. The fluid from a suspected lesion is allowed to flow into a capillary tube about 8 cm. long and 1 mm. in diameter. The two ends of this tube are sealed by pressing them into a soft paraffin-petrolatum mixture (50 percent of each). At the examining laboratory the serum is transferred to a slide by pressing one end of the capillary tube into a paraffin-petrolatum mixture until the plug in the opposite end is forced out. Such material must be examined within a few days after collection.

25. Genus Leptospira

25-1. *Bergey's Manual* (7th edition) lists only two species of *Leptospira*, although many serotypes are found worldwide in distribution. In the current scientific literature many of these serotypes have been given species names, although no formal classification has yet been established. At least a dozen serotypes of the leptospira cause disease in man. We will mention three of the more prominent of these: *Leptospira icterohemorrhagiae*, *L. canicola*, and *L. pomona*.

25-2. Pathogenic leptospiras are generally parasites of lower animals, both domestic and wild. Humans are infected through direct contact with infected tissues or body fluids, e.g., urine. Domestic animals such as hogs and cattle serve as a source of infection for slaughterhouse workers and farmers who process their own meat. The disease is often listed as being occupational. The most familiar disease is "Weil's disease," or infectious jaundice. Less serious infections are known as "field fever" or "swamp fever."

25-3. *Leptospira icterohemorrhagiae* is the causative agent of Weil's disease, a disorder encountered worldwide. In the United States it is only occasionally seen, and only about half of the persons infected develop the characteristic clinical jaundiced state. The leptospira are distributed throughout the body, and after about a week they may be found in the urine. Morphologically, *Leptospira icterohemorrhagiae* are thin, flexible, tightly coiled organisms. The spirochetes

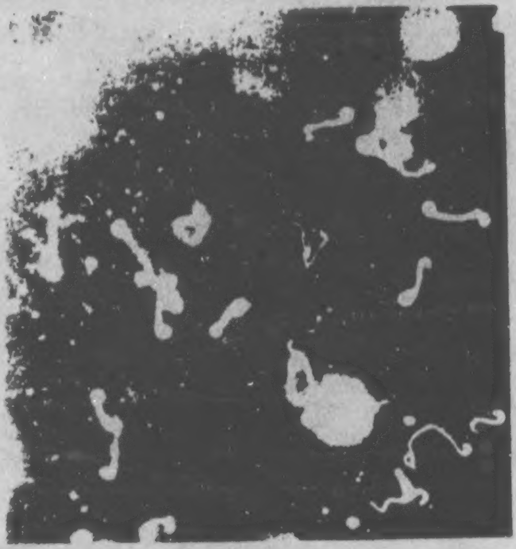


Figure 28. *Leptospira* as viewed with darkfield.

Bibliography

Books

BAILEY, W. R., and SCOTT, E. H. *Diagnostic Microbiology*. 2d Ed., St. Louis: C. V. Mosby Co., 1966.

BREED, Robert S., MURRAY, E.G.D., and SMITH, N.R. *Bergey's Manual of Determinative Bacteriology*. 7th Ed., Baltimore, Md.: The Williams and Wilkins Co., 1957.

BURROWS, Wm. *Textbook of Microbiology*. 18th Ed., Phila.: W.B. Sanders Co., 1963.

CRUICKSHANK, Robert, DUGUOD, J.P., and SWAIN, R.H.A. *Medical Microbiology*. 11th Ed., Baltimore, Md.: The Williams and Wilkins Co., 1965.

DORLAND, W.A.N. *The American Illustrated Medical Dictionary*. 21st Ed., Phila.: W.B. Sanders Co., 1947.

DUBOS, Rene' J., and HIRSCH, James G. *Bacterial and Mycotic Infections of Man*. 4th Ed., Phila.: J.B. Lippincott Co., 1965.

EDWARDS, P.R., and EWING, W.H. *Identification of Enterobacteriaceae*. 2d Ed., Minneapolis: Burgess Pub., 1962.

FROBISHER, Martin. *Fundamentals of Microbiology*. 7th Ed., Phila.: W. B. Sanders Co., 1962.

GOODMAN, Louis S., and GILMAN, A. *Pharmacological Basis of Therapeutics*. 3d Ed., New York: MacMillan, 1965.

JAWETZ, Ernest, MELNICK, J. L., and ADELBERG, E. *Review of Medical Microbiology*. 6th Ed., Los Altos, Calif.: Lange Med. Pub., 1966.

ROSEBURY, T. *Microorganisms Indigenous to Man*. New York: McGraw-Hill, 1962.

SALLE, A. J. *Fundamental Principles of Bacteriology*. 5th Ed., New York: McGraw-Hill, 1961.

SCHAUB, Isabelle, FOLEY, M. K., SCOTT, E. G., and BAILY, W. R. *Diagnostic Bacteriology*. 5th Ed., St. Louis: The C. V. Mosby Co., 1958.

SMITH, David T., CONANT, N. F., and OVERMAN, J. R. *Zinsser Microbiology*. 13th Ed., New York: Appleton-Century-Crofts, 1964.

SMITH, Lorraine. *Principles of Microbiology*. 5th Ed., St. Louis: The C. V. Mosby Co., 1965.

WILSON, Sir Graham, and MILES, A. A. *Topley and Wilson—Principles of Bacteriology and Immunity*. 5th Ed., Vol I and II, Great Britain: The Williams and Wilkins Co., 1964.

Periodicals

COLTEY, Roger W. "Laboratory Diagnosis of Streptococcal Infections." *American Journal of Medical Technology* (December 1965).

COLTEY, Roger W. "Identification of Small Gram Negative Coccoid Organisms Mima, Herellea, and Moraxella." *American Journal of Medical Technology* (1968), 1.29–32.

ELLNER, Paul D., and ELBOGEN, S. "Modern Methods in Tuberculosis Bacteriology for the General Hospital." *American Journal of Clinical Pathology*. Vol 48, No. 4 (October 1967), p. 435–440.

ELSTON, Harry R., and SALISBURY, W. A. "Recognition and Identification of the Genus Herellea, B. anitratum, and B5W." *American Journal of Medical Technology*, (May-June 1965).

HENRIKSEN, S. D. "Moraxella, Classification and Taxonomy." *Journal of General Microbiology* (1952), 6,318–328.

LIU, P. V. "A Modification of Elek's Technique on Micro Slide." *American Journal of Clinical Pathology* (1961), 36,471–473.

MURRAY, R. G. E., and TRUANT, J. P. "The Morphology, Cell Structure and Taxonomic Affinities of the Morexella." *Journal of Bacteriology* (1954), 67,13–22.

Department of the Air Force Publications

AFM 160-52, *Laboratory Procedures in Clinical Bacteriology*, 1 September 1962.

Other Government Publications

AIKEN, M. A., WARD, M. K., and KING, E. O. "A Study of a Group of Gram Negative Bacteria Resembling the Tribe Mineae" (BeBord). *U.S. Public Health Laboratory*, (1956), 14,126–136.

KUBICA, George P., and DYE, W. E. "Laboratory Methods for Clinical and Public Health: Mycobacteriology." *U.S. Department of Health, Education, and Welfare*, Public Health Service, Atlanta, Ga. (1967).

Commercial Manuals

"Anaerobic Microorganisms," *Microbiology Workshop Manual*, Schools of Laboratory Sciences, University Hospital and Clinic, University of Alabama Medical Center, Birmingham, Ala., June 4 and 5, 1965.

"Mycobacteria: Tuberculosis, Classified and Unclassified Acid Fast Bacilli," *Microbiology Workshop Manual*, Schools of Laboratory Sciences, University Hospital and Clinic, University of Alabama Medical Center, Birmingham, Ala., June 3 and 4, 1966.

NOTE: None of the items listed in the bibliography above are available through ECI. If you cannot borrow them from local sources, such as your base library or local library, you may request one item at a time on a loan basis from the AU Library, Maxwell AFB, Alabama, ATTN: ECI Bibliographic Assistant. However, the AU Library generally lends only *books* and a limited number of *AFM's*. TO's, classified publications, and other types of publications are *not* available. (For complete procedures and restrictions on borrowing materials from the AU Library, see the latest edition of the *ECI Catalog*.)

AU GAFS,ALA.(753205)2200

10-3 245

90412 02 21

WORKBOOK

MEDICAL LABORATORY TECHNICIAN–MICROBIOLOGY

SELF-TEACHING AIDS
CRE
VRE
SRG

This workbook places the materials you need *where* you need them while you are studying. In it, you will find the Study Reference Guide, the Chapter Review Exercises and their answers, and the Volume Review Exercise. You can easily compare textual references with chapter exercise items without flipping pages back and forth in your text. You will not misplace any one of these essential study materials. You will have a single reference pamphlet in the proper sequence for learning.

These devices in your workbook are autoinstructional aids. They take the place of the teacher who would be directing your progress if you were in a classroom. The workbook puts these self-teachers into one booklet. If you will follow the study plan given in "Your Key to Career Development," which is in your course packet, you will be leading yourself by easily learned steps to mastery of your text.

If you have any questions which you cannot answer by referring to "Your Key to Career Development" or your course material, use ECI Form 17, "Student Request for Assistance," identify yourself and your inquiry fully and send it to ECI.

Keep the rest of this workbook in your files. Do not return any other part of it to ECI.

EXTENSION COURSE INSTITUTE

Air University

246

TABLE OF CONTENTS

Study Reference Guide

Chapter Review Exercises

Answers to Chapter Review Exercises

Volume Review Exercise

ECI Form No. 17

STUDY REFERENCE GUIDE

1. *Use this Guide as a Study Aid.* It emphasizes all important study areas of this volume.

2. *Use the Guide as you complete the Volume Review Exercise and for Review after Feedback on the Results.* After each item number on your VRE is a three digit number in parenthesis. That number corresponds to the Guide Number in this Study Reference Guide which shows you where the answer to that VRE item can be found in the text. When answering the items in your VRE, refer to the areas in the text indicated by these Guide Numbers. The VRE results will be sent to you on a postcard which will list the *actual VRE items you missed.* Go to your VRE booklet and locate the Guide Number for each item missed. List these Guide Numbers. Then go back to your textbook and carefully review the areas covered by these Guide Numbers. Review the entire VRE again before you take the closed-book Course Examination.

3. *Use the Guide for Follow-up after you complete the Course Examination.* The CE results will be sent to you on a postcard, which will indicate "Satisfactory" or "Unsatisfactory" completion. The card will list *Guide Numbers* relating to the questions missed. Locate these numbers in the Guide and draw a line under the Guide Number, topic, and reference. Review these areas to insure your mastery of the course.

Guide Number	*Guide Numbers 200 through 222*
200	Introduction to the Gram-Positive Organisms; The Staphylococci, pages 1-3
201	The Streptococci, pages 3-7
202	Diplococcus Infections of Man, pages 7-9
203	The Corynebacteria, pages 9-13
204	Listeria Infections; Erysipelothrix Infections, pages 13-15
205	Bacillus Anthracis, pages 15-17
206	The Clostridia, pages 17-19
207	Introduction to the Gram-Negative Cocci and Coccoid Forms; Neisseria Infections of Man, pages 20-24
208	The Genus Veillonella; The Genera Mima, Herellea, and Moraxella; The Pleuro-Pneumonia-Like Organisms, pages 24-26
209	Introduction to the Small Gram-negative Bacilli; Pasteurella and Francisella, pages 27-29
210	Hemophilus and Bordetella, pages 29-33
211	The Genus Brucella, pages 33-35
212	Miscellaneous Gram-negative Bacilli, pages 35-37
213	Introduction to the Enteric Organisms; Classification and General Characters, pages 38-40
214	The Enterobacteriaceae: Shigella-Escherichia Division; Salmonella-Arizona-Citrobacter Division, pages 40-44
215	The Enterobacteriaceae: Klebsiella-Enterobacter-Serratia Division; Proteus-Providence Division, pages 44-46
216	Related Gram-negative Bacilli, pages 46-49
217	The Acid-Fast Bacilli; The Agents of Leprosy and Tuberculosis, pages 50-52
218	Distinguishing Characteristics of Tubercle Bacilli, pages 52-54
219	Special Diagnostic Tests, pages 54-56
220	Introduction to the Spirochetes; The Genus Borrelia, pages 57-59
221	The Genus Treponema, pages 59-64
222	Genus Leptospira, pages 64-65

CHAPTER REVIEW EXERCISES

The following exercises are study aids. Write your answers in pencil in the space provided after each exercise. Immediate after completing each set of exercises, check your responses against the answers for that set. Do not submit your answers to ECI for grading.

CHAPTER 1

Objectives: To learn to isolate, differentiate, and identify the various Gram-positive cocci routinely found in clinical specimens, and to show a knowledge of how and when to use special test procedures to aid in identification.

1. Why are staphylococci the most frequently isolated organisms in bacteriology? (1-1)

2. *Staphylococcus aureus* belongs to what family? (1-1)

3. What causes staphylococcal food poisoning? (1-3)

4. What are the oxygen requirements for the best growth of characteristic colonies of *Staphylococcus* spp.? (1-5)

5. What vitamins must be present to cultivate *Staphylococcus aureus* on synthetic media? (1-5)

6. Why is sodium chloride incorporated into mannitol-salt-agar? (1-8)

7. What is the principle of the catalase test? (1-10)

8. What is staphylokinase? (1-11)

9. Why must the coagulase test be checked at 30-minute intervals? (1-11)

10. An organism that divides in three planes during reproduction to form "packets" of eight cells most likely belongs to what genus? (1-13)

11. In phage typing, what is another name for the clear area formed as a result of the added bacteriophage? (1-15)

12. What phage types of *Staphylococcus aureus* have been associated with outbreaks of "hospital staph" infections? (1-16)

13. In the family Lactobacillaceae, which two genera are of medical importance to bacteriologists? (2-2)

14. What enrichment is essential for a medium to support the growth of most pathogenic streptococci? (2-5)

15. Which colony characteristic of *Streptococcus* spp. gives an indication of pathogenicity and offers the best tentative means of separating types? (2-6)

16. How does alpha hemolysis differ from beta hemolysis? (2-6)

17. What is streptokinase? (2-8)

18. The skin rash of scarlet fever is a result of the production of what type toxin by streptococci? (2-8)

19. Of the several schemes for classifying the streptococci, two of the more widely used are those devised by Lancefield and Sherman. What is the basic difference between the two schemes? (2-9)

20. Which of the two methods for classifying *Streptococcus* spp. is based on the antigenic structure of the organism? (2-9)

21. The letters A through O when used with the streptococci name indicate that the organism was classified according to which scheme? (2-10)

22. Beta hemolytic streptococci are generally classified by the Sherman method as what group? (2-11)

23. What separates members of the Sherman enterococcus group from members of the other groups? (2-12)

24. The Bacitracin screening technique would show that the organisms are sensitive (susceptible) to Bacitracis if they belong to which Lancefield group? (2-15)

25. SF broth is used to grow organisms belonging to which Lancefield group? (2-16)

26. What is the principle of using sodium hippurate broth to differentiate between Lancefield's group B from A, C, and D? (2-16)

27. Small Gram-positive, slightly elongated cocci arranged in pairs most likely belong to what genus? (3-4)

252

28. When grown on blood agar in a candle jar, typical virulent strains of pneumococci form what kind of colonies? (3-7)

29. Virulence of *Diplococcus pneumoniae* is, in part, dependent upon what section of the bacterial cell? (3-5, 8)

30. When describing the colonies of *D. pneumoniae*, what do the letters M, S, and R stand for? (3-8)

31. What are two tests that can be used to help identify *D. pneumoniae?* (3-9)

32. What is the Neufeld Quellung reaction? (3-10)

33. What are the three varieties of medical importance of *Corynebacterium diphtheriae?* (4-1)

34. What is another term that is used in referring to *Corynebacterium diphtheriticum?* (4-1)

271

35. How are the *Corynebacterium diphtheriae* organisms spread? (4-2)

36. When attempting to confirm a diagnosis of diphtheria what is the specimen of choice? (4-3)

37. What causes the pleomorphism that you see on examining a Gram stain of corynebacterium? (4-4)

38. Which two agars are recommended for the cultivation of *C. diphtheriae?* (4-6)

39. How can the species *Corynebacterium diphtheriae* be identified with certainty? (4-9)

40. For what is Klebs-Loeffler (KL) medium used? (4-10)

41. Which anaerobic diphtheroid is often confused with *Actinomyces?* (4-11)

42. What is the etiologic agent of "glandular fever" of children? (5-1, 2)

43. *Listeria monocytogenes* belongs to what family? (5-2)

44. Which tests help differentiate *Listeria monocytogenes* from the diphtheroids? (5-6)

45. *Erysipelothrix insidiosa* belongs to what family? (6-1)

46. Because *Erysipelothrix insidiosa* grows poorly on simple media, what must be added to enhance its growth? (6-3)

47. What makes *Bacillus megaterium* of importance in medical bacteriology? (7-1)

48. What is anthrax? (7-2)

49. Large Gram-positive, sporeforming rods are members of what genus? (7-6)

50. Members of the genus *Clostridium* belong to which family? (8-1)

51. Of the six antigenic types of *Cl. botulinum*, which ones usually affect man? (8-6)

52. Which one of the organisms *Cl. perfringens, Cl. septicum, Cl. novyi,* and *Cl. botulinum* is most likely to cause disease? (8-7)

53. Why is direct examination of suspected foods in cases of botulism of little value in identifying the organism? (8-12)

54. Suppose you are stationed at a small hospital or dispensary laboratory where there is no equipment available to make an anaerobic environment for petri dish cultures. The physician comes to you with a suspected case of gas gangrene. Under these circumstances how would you culture the specimen for *Clostridium* spp.? (8-13)

55. What is another name given to the double zone of hemolysis which may develop in *Clostridium perfringens* colonies? (8-14)

56. What is the best way to differentiate *Bacillus* spp. from *Clostridium?* ([illegible]-14, 17)

CHAPTER 2

Objectives: To learn to isolate, differentiate, and identify the various Gram-negative cocci and coccoid organisms which may be isolated from various clinical specimens; to show a knowledge of how to use and interpret the various tests necessary for the identification of these organisms.

1. Which two *Neisseria* species are human pathogens? (9-1)

2. Why is the incidence of gonorrheal conjunctivitis of infants so low in the United States? (9-2)

3. What is the major portal of entry for meningococci? (9-3)

4. Of the four serological groups, which contains the majority of meningococci responsible for epidemics? (9-3)

5. If no urethral discharge is present in a male suspected of having gonorrhea, how can a diagnosis best be made? (9-7)

257

6. To determine whether or not gonorrhea is present in a female, where should a specimen be collected for culture? (9-7)

7. To cultivate *N. gonorrhoeae* or *N. meningitidis*, chocolate agar must be supplemented with one of which two ingredients? (9-9)

8. Why is the Thayer-Martin medium such a good medium to use for culturing the meningococci and the gonococci? (9-10)

9. What is the optimum growth temperature for pathogenic *N. gonorrhoeae?* (9-11)

10. Which of *N. flavescens*, *N. subflava*, *N. perflava*, and *N. gonorrhoeae* does not usually produce a yellow or yellow-green pigment? (9-12)

11. Why should carbohydrate fermentation studies be performed under normal atmospheric conditions and not under increased CO_2? (9-13)

12. The oxidase test is used to determine the presence of what? (9-14)

13. How can confirmation of a *Neisseria* species be accomplished? (9-14)

14. Although Bergey's Manual (7th ed.) lists six species of *Veillonella*, which one has been more clearly characterized? (10-1)

15. What are the oxygen requirements of members of the genus *Veillonella?* (10-1, 3)

16. To which tribe do the genera *Mima*, *Herellea*, and *Moraxella* belong? (11-1)

17. Which of the mimae is most easily confused with the neisseriae? (11-2)

18. What is the name of the one recognized species of the genus *Herellea?* (11-3)

19. How can members of the genus *Herellea* be differentiated between *Neisseria* spp. and the *Mima* and *Moraxella* spp.? (11-3, 6)

20. Why can MacConkey and EMB agar be used to differentiate between *Neisseria* spp. and the *Mima* and *Herellea* spp.? (11-6)

21. In order to cultivate members of the genus *Mycoplasma*, what ingredients should be added to media? (12-3)

22. Recognition of the PPLO group is generally based on what? (12-4)

23. With what organisms are "mulberry" type colonies generally associated? (12-4)

24. Identification of the colonies of the PPLO is best done by using which staining technique? (12-5)

25. Are the stains decolorized by PPL organisms when special staining techniques are used? (12-5)

CHAPTER 3

Objectives: To learn to isolate, differentiate, and identify the various small Gram-negative bacilli found in clinical specimens and to show a knowledge of how to use the various biochemical test procedures needed for identification of these organisms.

1. What is the causative agent of human plague? (13-1)

2. What is the most likely cause of hemorrhagic septicemia in man? (13-2)

3. If plague is suspected, what type of specimen should be submitted for culture? (13-4, 5)

4. More abundant growth at room temperature than at 37° C. helps identify which *Pasteurella* spp.? (13-8)

5. Cultivation of *Francisella tularensis* is best accomplished by using which medium? (13-10)

6. Primary growth of *Fr. tularensis* from blood cultures requires how many days? (13-10)

7. For growth on laboratory media, *Hemophilus* species must have one or the other, or both, of what two compounds? (14-2)

8. Of the X and V factors, which is heat stable and which is heat labile? (14-2)

9. When attempting to isolate *Hemophilus* species, why is sheep blood *not* recommended? (14-5)

10. Why is the V factor added after blood agar has been co[illegible] chocolate agar? (14-5)

11. If enrichment supplements are not available, how can blood agar plates be used to cultivate *Hemophilus* species? (14-6)

12. What is meant by "satellitism"? (14-6)

13. In what type of colonies are capsules most likely to be seen from cultures of *H. influenzae?* (14-8)

14. What is the etiologic agent of "pink eye"? (14-8)

15. What is the etologic agent of "chancroid"? (14-9)

16. How may the five species of *Hemophilus* that we have studied be best differentiated? (14-11)

17. How do members of the genus *Bordetella* differ from those of the genus *Hemophilus?* (14-13)

18. Describe *Bordetella pertussis* as to their sporeforming and motility ability. (14-15)

19. What three major ingredients are found in Bordet-Gengou media? (14-16)

20. Cough plates should be incubated and examined after how many days? (14-16)

21. Mercury drop or bisected pearl colonies on glycine-potato-blood agar are descriptive of which organism? (14-16)

22. What four tests can be used to help differentiate *B. pertussis* from the other two species? (14-18)

23. What causes the brownish hemolysis often seen surrounding colonies of *B. parapertussis* when grown on Bordet Gengou medium? (14-18)

24. Undulant fever is caused by members of what genus? (15-1)

25. How does man generally become infected with brucellosis? (15-2)

26. What is the specimen of choice for isolating brucella organisms from an infected person? (15-5)

27. Which of the *Brucella* spp. can only be cultivated in an atmosphere containing 5 to 10 percent CO_2? (15-5)

28. How may the three colony types (S, R, M) of *Brucella* spp. be easily identified? (15-6)

29. Urea is hyrolyzed rather rapidly by which species of *Brucella*. (15-8)

264

30. *Actinobacillus mallei* is the etiologic agent for what disease? (16-2)

31. A physician sends a male patient to the laboratory for a darkfield examination. The patient has a history of recent sexual contact and an ulcerative lesion of the genital area. The darkfield examination is negative. The physician says that it may be a case of granuloma inguinale and he wants you to run an examination for the etiologic agent. What is the name of the organism and how would you perform the examination? (16-6-8)

32. What is the L form of *Streptobacillus moniliformis?* (16-11)

CHAPTER 4

Objectives: To know how to isolate, differentiate, and identify the various enteric organisms found in clinical specimens, and to show a knowledge of how to use and interpret the various biomedical tests needed for the proper identification of the enteric organisms.

1. Most enteric organisms belong to what family? (17-2, 3)

2. What tests are used to determine the IMViC reaction? (17-5)

3. Why are stool specimens inoculated to an enrichment broth such as selenite F or tetrathionate? (17-8)

283

4. Are MacConkey, *Salmonella-Shigella*, bismuth sulfite, and desoxycholate all considered selective media? If not, which are not? (17-9)

5. How do eosin-methylene blue and MacConkey agars provide for the differentiation of lactose and non-lactose fermenting organisms? (17-9)

6. Which of the following is found in TSI agar and not in Kligler's iron agar: lactose, sucrose, glucose, or an iron compound? (17-10, 11)

7. Of *Vibrio cholerae*, *Salmonella* spp., and *Shigella* spp., which requires a more alkaline medium in order to be cultivated? (17-15)

8. How many enterics produce spores under the right conditions? (17-15)

9. What type organisms are found in the *Alkalescens-Dispar* (A-D) group? (18-2)

10. What is there about *Shigella dysenteriae* that makes infections with it so serious? (18-4)

11. Nonlactose fermenting *E. coli* can be confused with which organisms? (18-7)

12. What color indicates an alkaline reaction at the slant butt junction of an acid citrate-mannitol agar? (18-7)

13. Of *Escherichia coli, Alkalescense-Dispar* group, *Shigella* spp., and *Escherichia coli,* which gives a positive citrate-mannitol agar reaction? (18-8)

14. Organisms of the *Salmonella-Arizona-Citrobacter* division are separated taxonomically from the *Shigella-Escherichia* division on the basis of which tests? (18-9)

15. Which two tests help separate the *Proteus-Providence* division from the other division? (18-9)

16. Blood cultures taken for the purpose of detecting enteric fevers are usually of value when taken how long after the patient becomes infected? (18-11)

17. Which of the *Salmonella* species is most often found as the cause of gastroenteritis in man? (18-12)

18. *Paracolobactrum intermedium* is commonly referred to as what? (18-14)

19. How can *Citrobacter* be differentiated from *Escherichia coli?* (18-14)

20. Members of the genus *Edwardsiella* were earlier referred to as what? (18-15)

21. What is also known as Friedlander's bacillus? (18-18)

22. The nonmotile strains of the genus *Aerobacter* are now considered under which name? (18-19)

23. What new generic name is given for the motile strain of *Aerobacter* spp.? (18-19)

24. The *Hafnia* group of enterics is a subgroup of which genus? (18-19)

25. Why is the genus *Serratia* of importance if it is a saprophyte generally found in soil, water, milk, and food? (18-20)

26. How can you prevent the swarming phenomenon of *Proteus* spp. on media? (18-22)

27. What organism is generally associated with "Summer diarrhea" of children? (18-23)

28. Although members of the genus *Proteus* and the Providence group are closely related, they can be easily separated on the basis of what simple test procedure? (18-24, 25)

29. A negative sucrose and lactose reaction with a positive glucose reaction in a tube of triple sugar iron agar results in what? (18-26)

30. What is the Weil-Felix reaction? (18-27)

31. Why can't serological grouping be relied upon to distinguish between the genera *Proteus, Salmonella,* and *Escherichia?* (18-28)

32. How is glucose fermentation used to differentiate members of the family Achromobacteraceae from the family Enterobacteriaceae? (19-2)

33. Of *Alcaligenes fecalis*, *Escherichia coli*, ***Pseudomonas aeruginosa***, and *Edwardsiella tarda*, which belongs to the family Achromobacteraceae? (19-2)

34. Entericlike organisms which produce a water soluble pigment and show an alkaline throughout TSI reaction probably belong to what genus? (19-4)

35. What is the genus and species name of an organism of the family Spirillaceae that causes one form of "rat-bite fever"? (19-8)

36. Morphological cells of the genus *Vibrio* usually appear in what form? (19-8, 11)

37. If you are to Gram-stain feces for the vibrio organisms, which part of the stool should be picked. (19-10)

CHAPTER 5

Objectives: To learn to isolate, differentiate, and identify the more common acid-fast bacilli found in clinical specimens and to show a knowledge of how to use and interpret the various biomedical tests needed to properly identify the various acid-fast bacilli.

1. To which family does the genus *Nocardia* belong? (Intro.-2)

2. What acid-fast organism can be easily isolated from the human mouth? (Intro.-4)

3. Hansen's bacillus is another name for which mycobacterium? (20-1)

4. Besides *Mycobacterium tuberculosis*, what organism is a principal cause of human tuberculosis? (20-5)

5. How is bovine tuberculosis usually transmitted to man? (20-6)

6. Bloodstream dissemination of the tubercle bacilli can result in what kind of tuberculosis? (20-7)

7. Identify the Runyon classification. (20-10)

8. The mycobacteria which form a yellow pigment in the presence of light are placed in which Runyon Group? (20-11)

9. The "Battey bacilli" is a term applied to the mycobacteria of which Runyon Group? (20-12)

10. Of *M. phlei*, *M. kansasii*, *M. scrofulaceum*, and *M. fortuitum*, which is generally considered to be a saprophyte? (20-11-13)

11. Which of the mycobacterium is commonly called the "timothy grass bacillus"? (20-13)

12. According to the National Tuberculosis Association, how should an AFB smear be reported if ten or more cells per slide are seen? (21-3)

13. According to the National Tuberculosis Association, if less than three positive fields are found on an AFB slide, what should you do? (21-3)

14. Describe the colony texture appearance of the typical human tubercle bacillus on Petragmani's media. (21-4)

15. The atypical group of mycobacteria can usually be differentiated from the human, avian, and bovine species on the basis of growth at what temperature? (21-6)

16. What three media are recommended for the cultivation of the mycobacteria? (21-7, 8)

17. What are the principal growth constituents found in the enriched media usually used to cultivate the mycobacteria? (21-7)

18. What are the advantages of using Middlebrook 7H10 agar over Petragnani's agar? (21-8)

19. How can the pathogenicity of the mycobacteria be best determined? (22-1)

20. What is the name of the test used to detect formation of nicotinic acid? (22-2)

21. When testing for niacin production and aniline and cyanogen bromide reagents are added, what color is found in a positive test? (22-3)

22. With reference to the catalase reaction, what can be said of all of the acid-fast bacilli studied? (22-4)

23. A medium containing tripotassium phenolphthalein disulfate is used to perform which biochemical test for differentiating the mycobacteria? (22-6)

24. What is the minimum number of acid-fast bacilli that must be seen on a direct smear in order to do an accurate, direct antibiotic susceptibility test? (22-9)

25. What is used as the basis for determining the dilution factor to be used for testing acid-fast organisms' antibiotic susceptibility? (22-9, 10)

26. Which three drugs are usually used for the initial antibiotic susceptibility test? (22-10)

CHAPTER 6

Objective: To isolate, differentiate, and identify the various spirochete forms of bacteria found in the clinical laboratory, and to show a knowledge of how and when to use special tests to aid in their identification.

1. The diseases yaws and relapsing fever are caused by organisms belonging to what family? (Intro.-1, 5)

2. What are the three distinguishing properties of the genera found in the family Treponemataceae? (Intro.-6)

3. Which of the *Borrelia* spp. causes ulcerative stormatitis? (23-1)

274

4. Which of the three genera of the family Treponemataceae can be easily stained with the Gram stain technique? (23-4)

5. To cause the disease "trench mouth," a fusiform bacilli must be present with what organism? (23-7, 9, 10)

6. Yaws is caused by what organism? (24-1)

7. During which of the three stages of syphilis does the chancre usually first appear? (24-3)

8. How is primary syphilis generally diagnosed? (24-3)

9. During the secondary stage of syphilis where may the spirochetes of *T. pallidum* be isolated? (24-4)

10. Describe the organism *Treponema pallidum*. (24-5)

293

11. Why is the spirochete of *Treponema pallidum* difficult to stain with Gram stain? (24-6)

12. Why should you have a brief interview with a patient prior to performing a darkfield examination? (24-10)

13. "Weil's disease" is caused by members of what genus? (25-2)

14. What is the major characteristic peculiar to the spirochete of *Leptospira icterohemorrhagiae?* (25-3)

15. Which of the three genera of the family Treponemataceae can be cultivated artificially? (23-6; 24-7; 25-6)

16. For cultivation of *L. icterohemorrhagiae* what specimens are required? (25-6)

17. At what time period during infection with *L. icterohemorrhagiae* do serological tests yield the best results? (25-6)

18. Although *L. canicola* produces an infection similar to that of *L. icterohemorrhagiae*, why is it often not diagnosed? (25-7)

277

ANSWERS FOR CHAPTER REVIEW EXERCISES

CHAPTER 1

1. They are found in the air, on dust particles, and on the body. (1-1)
2. Micrococcaceae. (1-1)
3. Ingesting food that contains significant amounts of the heat stable exotoxin produced by *Staphylococcus* spp. The toxin is rapidly absorbed by the intestinal mucosa, resulting in acute symptoms. (1-3)
4. Characteristic colonies grow best under aerobic conditions, but growth will take place in an anaerobic environment if hydrogen and uracil are present. (1-5)
5. Thiamin and nicotinic acid. (1-5)
6. A high concentration of sodium chloride in mannitol-salt-agar is inhibitory to most organisms. (1-8)
7. Catalase liberates oxygen from hydrogen peroxide. (1-10)
8. Staphylokinase is an enzyme produced by some staphylococci and has the ability to dissolve clots. (1-11)
9. To prevent reporting of false negative reactions. (1-11)
10. *Sarcina* spp. (1-13)
11. Plaque. (1-15)
12. Phage types 80 and 81. (1-16)
13. *Streptococcus* and *Peptostreptococcus.* (2-2)
14. Blood. (2-5)
15. Hemolysis (alpha and beta). (2-6)
16. With beta hemolysis there is complete destruction of the red blood cells immediately surrounding the colony. With alpha hemolysis, the zone of hemolysis has irregular edges and may contain partially destroyed red blood cells. (2-6)
17. Streptokinase is an enzyme produced by some streptococci and is capable of breaking down fibrin clots. (2-8)
18. Erythrogenic toxins. (2-8)
19. The Lancefield classification is based on the antigenic structure of the organisms. The composition of a carbohydrate antigen—"C" substance—is different for each immunologic group. (2-9)
20. Lancefield. (2-9)
21. Lancefield. (2-10)
22. Pyogens. (2-11)
23. Members of the enterococcus group grow well in a broth containing a high salt content. (2-12)
24. Group A. (2-15)
25. Group D. (2-16)
26. Sodium hippurate is hydrolyzed to benzoic acid. Benzoic acid is then tested for using 12 percent ferric chloride solution. (2-16)
27. *Diplococcus.* (3-4)

28. Flat, smooth, slimy, and transparent colonies are formed. (3-7)

29. The capsule. (3-5, 8)

30. M = mucoid; S = smooth; and R = rough. (3-8)

31. Sodium desoxycholate (or bile) and optochin (or Taxo P®). (3-9)

32. The Neufeld-Quellung reaction is a procedure whereby an organism is exposed to a type-specific antisera and the organism reacts by swelling. (3-10)

33. *Gravis, intermedius,* and *mitis.* (4-1)

34. *Corynebacterium hoffmanii.* (4-1)

35. By nasal droplets, oral spray, or by direct contact. (4-2)

36. Material from lesions and/or pseudomembranes in the throat usually yield large numbers of the characteristic bacilli. (4-3)

37. Irregular distribution of cytoplasmic granules. (4-4)

38. Loeffler's serum slants and potassium tellurite agar. (4-6)

39. By demonstrating the production of toxins. (4-9)

40. For in-vitro virulence testing. (4-10)

41. *C. acnes.* (4-11)

42. *Listeria monocytogenes.* (5-1, 2)

43. Corynebacteriaceae. (5-2)

44. Catalase, motility, and metachromatic granules. (5-6)

45. Corynebacteriaceae. (6-1)

46. Serum or glucose. (6-3)

47. *B. megaterium* is a saprophyte which closely resembles *B. anthracis.* (7-1)

48. Anthrax is primarily a disease of herbivorous animals. For man it is an occupational disease found most often in persons dealing with infected animals. (7-2)

49. *Bacillus.* (7-6)

50. Bacillaceae. (8-1)

51. Types A and E. (8-6)

52. *Cl. perfringens.* (8-7)

53. Direct examination is of little value because very few organisms are ordinarily present in such specimens. (8-12)

54. Inoculate the lower portion of a tube of thioglycollate medium containing 0.6 percent glucose with the specimen and incubate at 37° C. As you know, this medium should be available at any size laboratory. This medium affords the nutritive and anaerobic requirements for *Clostridium* spp. At 37° C. the organisms grow well in the depths of the thioglycollate medium. (8-13)

55. A "target." (8-14)

56. Culture the organisms aerobically and anaerobically. *Bacillus* spp. and *Clostridium* spp. are both Gram-positive rods; however, *Bacillus* spp. are aerobes whereas *Clostridium* spp. are obligate anaerobes. (8-14, 17)

CHAPTER 2

1. *N. gonorrhoeae* and *N. meningitidis*. (9-1)
2. Because of the legal requirement that as a preventive measure suitable medication be applied to the eyes of all newborn. (9-2)
3. Nasopharynx. (9-3)
4. Group A. (9-3)
5. By collecting and culturing the first 5 to 10 ml. of a morning urine. (9-7)
6. From the cervix or urethra. (9-7)
7. Yeast or liver extract. (9-9)
8. Thayer-martin medium greatly suppresses saprophytic *Neisseria* species. Also, *Mima* organisms, which often are confused for the gonococci, are suppressed. (9-10)
9. 35° to 36° C. (9-11)
10. *N. gonorrhoeae*. (9-12)
11. Absorption of CO_2 by the carbohydrate medium is accompanied by a drop in pH, resulting in false positive reactions. (9-13)
12. Cytochrome oxidase. (9-14)
13. By the aid of carbohydrate reactions. (9-14)
14. *Veillonella alcalescens*. (10-1)
15. Anaerobes. (10-1, 3)
16. Tribe Mimeae. (11-1)
17. *Mima polymorpha* var. *oxidans*. (11-2)
18. *Herellea vaginicola*. (11-3)
19. Members of the genus *Herellea* oxidize xylose, whereas *Mima* and *Moraxella* do not. (11-3, 6)
20. Most *Neisseria* spp. fail to grow, whereas *Mima* and *Herellea* species will grow on this agar. (11-6)
21. 20 to 30 percent ascitic fluid or 10 percent horse serum. (12-3)
22. Colony characteristics. (12-4)
23. PPLOs. (12-4)
24. Dienes' staining method. (12-5)
25. PPL organisms do not decolorize the stain. (12-5)

CHAPTER 3

1. *Pasteurella pestis*. (13-1)
2. *Pasteurella multocida*. (13-2)
3. If bubonic plague is suspected, material aspirated from buboes should be submitted. If pneumonic plague is suspected, sputum should be submitted. Blood specimens may also be cultured in cases of suspected bubonic plague. (13-4, 5)

4. *P. pseudotuberculosis.* (13-8)
5. Cystine-glucose-blood agar. (13-10)
6. 4 to 7 days. (13-10)
7. Hemin (X factor) and phospho-pyridine nucleotide (V factor). (14-2)
8. X factor is heat stable and V factor is heat labile. (14-2)
9. Sheep blood contains some factors which are inhibitory to some *Hemophilus* spp. (14-5)
10. The V factor, being heat labile, is destroyed at the temperature required to convert blood agar into chocolate agar. It must, therefore, be added as a supplement. (14-5)
11. By first streaking the blood agar plate surface with the suspected *Hemophilus* spp. and then making a single streak with *Staphylococcus* spp. (14-6)
12. The phenomenon of small colonies growing only in close proximity to another colony which provides the smaller colonies with the required growth factors is referred to as "satellitism." (14-6)
13. Mucoid. (14-8)
14. *Hemaphilus aegyptius.* (14-8)
15. *Hemophilus ducreyi.* (14-9)
16. On the basis of X and V factor requirements coupled with the presence or absence of hemolysis. (14-11)
17. *Bordetella* spp. organisms do not require X or V factor, whereas *Hemophilus* spp. requires X or V or both. (14-13)
18. Nonsporeforming and nonmotile. (14-15)
19. Glycine, potato, and 15 percent blood. (14-15)
20. 3 to 5 days. (14-16)
21. *Bordetella pertussis.* (14-16)
22. Citrate utilization, urease production, motility, and nitrate reduction. (14-18)
23. High concentration of copper-containing proteins. (14-18)
24. *Brucella.* (15-1)
25. By ingesting contaminated raw milk and improperly cooked meat from infected animals. (15-2)
26. Blood taken during the febrile stage. (15-5)
27. *Brucella abortus.* (15-5)
28. By using a 1:2,000 solution of crystal violet to stain the colonies. (15-6)
29. *Brucella suis.* (15-8)
30. Glanders disease. (16-2)
31. *Calymmatobacterium granulomatis* is the causative organism. Make a smear of the ulcerated lesion and stain it with Wright's stain. Look for blue bacilli surrounded by a well-defined, dense pink capsule inside of large mononuclear white cells. These intracellular organisms are reported as "Donovani bodies." The organisms cannot be isolated on ordinary culture media, but they can be grown in chick embryo tissues and then subcultured to artificial media such as beef-heart-infusion agar. (16-6-8)

32. The L form is a colonial variant where the colony grows into the medium and cannot usually be transferred with the laboratory loop. (16-11)

CHAPTER 4

1. Enterobacteriaceae. (17-2,3)
2. Indole, methyl red, Voges-Proskauer, and citrate. (17-5)
3. This permits certain enteric organisms, i. e., *Salmonella* spp. and *Shigella* spp. to multiply at the expense of nonpathogens. (17-8)
4. No, MacConkey is not a selective medium. (17-9)
5. Colors are based on a change of pH when acids are produced as a result of lactose fermentation. This change in pH affects the color of the indicator dye present in the agar. (17-9)
6. Sucrose. (17-10, 11)
7. *Vibrio cholerae.* (17-15)
8. None; all enterics are nonspore producers. (17-15)
9. The A-D category contains nonmotile organisms that are essentially slow lactose-fermenting *E. coli* strains; yet they possess the serological typeability of the shigellae. (18-2)
10. *Shigella dysenteriae*, in addition to producing a powerful endotoxin, also produces an exotoxin (neurotoxin). (18-4)
11. *Shigella* spp. and the A-D group. (18-7)
12. Blue color. (18-7)
13. *Escherichia coli.* (18-8)
14. The IMViC reaction and H_2S production. (18-9)
15. Urease and phenylalanine. (18-9)
16. Blood cultures are usually positive only during the first and second week of infection. (18-11)
17. *S. typhimurium.* (18-12)
18. The *Bethesda-Ballerup* Group. (18-14)
19. *Citrobacter* is an H_2S producer and is citrate positive. (18-14)
20. *Bartholemew* organisms. (18-15)
21. *Klebsiella pneumoniae.* (18-18)
22. *Klebsiella pneumoniae.* (18-19)
23. *Enterobacter.* (18-19)
24. *Enterobacter.* (18-19)
25. Members of the genus *Serratia* have been implicated in hospital infection epidemics and as a secondary invader in certain types of lung disease. (18-20)
26. By adding chloral hydrate to the media, or by using media lacking in sodium chloride, or by using media with 6 percent agar. (18-22)
27. *Proteus morganii.* (18-23)

28. Urease test. (18-24, 25)
29. An acid butt and alkaline slant. (18-26)
30. The Weil-Felix reaction is a name applied to the phenomenon of having certain strains of *Proteus* spp. react with the antibodies formed against a rickettsia. (18-27)
31. Because the similarity in O antigens can result in cross reactivity. (18-28)
32. Members of the family Achromobacteraceae attack glucose only under aerobic conditions, whereas members of the family Enterobacteraceae attack glucose anaerobically as well as aerobically. (19-2)
33. *Alcaligenes fecalis.* (19-2)
34. *Pseudomonas.* (19-4)
35. *Spirillum minus.* (19-8)
36. Curved or comma-shaped rods. (19-8, 11)
37. Mucus flecks. (19-10)

CHAPTER 5

1. Actinomycetaceae. (Intro.-2)
2. *Actinomyces israelii.* (Intro.-4)
3. *Mycobacterium leprae.* (20-1)
4. *M. bovis.* (20-5)
5. Through the consumption of raw milk or inadequately cooked meat from infected cattle. (20-6)
6. It can cause miliary, or "mustard seed," tuberculosis. (20-7)
7. The classification that uses cultural and morphological properties, animal studies, and metabolic traits to assign the mycobacteria to four subgroups. (20-10)
8. Photochromogens. (20-11)
9. Nonphotochromogens. (20-12)
10. *M. phlei.* (20-11-13)
11. *M. phlei.* (20-13)
12. "Few acid-fast bacilli seen." (21-3)
13. Prepare a new smear. (21-3)
14. Colonies are dry, nodular, and rough (cauliflower) in appearance. (21-4)
15. 25° C. (21-6)
16. Lowenstein-Jensen's, Petragnani's, and Middlebrook 7H10. (21-7, 8)
17. Egg, glycerol, asparagine, and potato starch. (21-7)
18. Middlebrook 7H10 medium is clear, so early detection of colonies is possible using the microscope; it allows easy separation of mixed colony types; and agar is not altered or destroyed by bacteria contamination. Also, growth occurs within 3 weeks, whereas 8 weeks are often required on Petragnani's medium. (21-8)

19. By inoculating guinea pigs, rabbits, or mice. (22-1)
20. The niacin test. (22-2)
21. Yellow. (22-3)
22. They are catalase-positive. (22-4)
23. Sulfatase. (22-6)
24. 10. (22-9)
25. Dilution is made according to the number of organisms seen on the smear under oil immersion. (22-9, 10)
26. Isoniazid (INH), para-aminosalicylic acid (PAS), and streptomycin. (22-10)

CHAPTER 6

1. Treponemataceae. (Intro.-1, 5)
2. Morphology, staining characteristics, and oxygen requirements. (Intro.-6)
3. *Borrelia vincentii.* (23-1)
4. *Borrelia.* (23-4)
5. *Borrelia vincentii.* (23-7, 9, 10)
6. *Treponema pertenue.* (24-1)
7. The primary stage. (24-3)
8. By the darkfield examination of the chancre. (24-3)
9. Skin lesions. (24-4)
10. A spirochete having a flexible cylindrical body measuring 5 to 16 microns in length and possessing eight to fifteen spirals is probably *Treponema* pallidum. (24-5)
11. The amount of protoplasm available for staining is so small that the necessary visual contrast is lacking. (24-6)
12. If the patient has treated his lesion with a germicidal agent, examination must be deferred until all germicide has been removed by washing and several hours have elapsed. (24-10)
13. *Leptospira.* (25-2)
14. The terminal third of the organism is quite flexible and often forms a hook. (25-3)
15. *Borrelia;* Nonvirulent Reiter strain of *T pallidum;* and *Leptospira.* (23-6; 24-7; 25-6)
16. Blood or urine. (25-6)
17. Between the seventh and fourteenth day. (25-6)
18. The disease produced by *L. canicola* is less severe and is rarely accompanied by jaundice. (25-7)

284

STOP-

1. MATCH ANSWER SHEET TO THIS EXERCISE NUMBER.

2. USE NUMBER 1 PENCIL.

90412 02 21

VOLUME REVIEW EXERCISE

Carefully read the following:

DO'S

1. Check the "course," "volume," and "form" numbers from the answer sheet address tab against the "VRE answer sheet identification number" in the righthand column of the shipping list. If numbers do not match, take action to return the answer sheet and the shipping list to ECI immediately with a note of explanation.

2. Note that numerical sequence on answer sheet alternates across from column to column.

3. Use only medium sharp # 1 black lead pencil for marking answer sheet.

4. Circle the correct answer in this test booklet. After you are sure of your answers, transfer them to the answer sheet. If you *have* to change an answer on the answer sheet, be sure that the erasure is complete. Use a clean eraser. But try to avoid any erasure on the answer sheet if at all possible.

5. Take action to return entire answer sheet to ECI.

6. Keep Volume Review Exercise booklet for review and reference.

7. If *mandatorily* enrolled student, process questions or comments through your unit trainer or OJT supervisor.
   If *voluntarily* enrolled student, send questions or comments to ECI on ECI Form 17.

DON'T

1. Don't use answer sheets other than one furnished specifically for each review exercise.

2. Don't mark on the answer sheet except to fill in marking blocks. Double marks or excessive markings which overflow marking blocks will register as errors.

3. Don't fold, spindle, staple, tape, or mutilate the answer sheet.

4. Don't use ink or any marking other than with a # 1 black lead pencil.

Note: The 3-digit number in parenthesis immediately following each item number in this Volume Review Exercise represents a Guide Number in the Study Reference Guide which in turn indicates the area of the text where the answer to that item can be found. For proper use of these Guide Numbers in assisting you with your Volume Review Exercise, read carefully the instructions in the heading of the Study Reference Guide.

Multiple Choice

Chapter 1

1. (200) Which of the following is *least* likely to be caused by *Staphylococcus aureus?*

   a. Pneumonia.
   b. Sinusitis.
   c. Otitis media.
   d. Osteomyelitis.

2. (200) Staphylococcal food poisoning is caused by an exotoxin that is

   a. heat labile.
   b. an endotoxin.
   c. heat stable.
   d. destroyed by refrigeration.

3. (200) Synthetic media used for the cultivation of *Staphylococcus* spp. must include

   a. catalase and mannitol.
   b. thiamin and nicotinic acid.
   c. mannitol and thiamin.
   d. nicotinic acid and mannitol.

4. (200) An organism that appears as Gram-positive cocci arranged in tetrads when stained from a grey, moist, and viscid colony on blood agar is most likely to be

   a. *Gaffkya* spp.
   b. *Sarcina* spp.
   c. *Streptococcus* spp.
   d. *Staphylococcus epidermidis.*

5. (200) Organisms of the genus *Staphylococcus* are

   a. nonmotile and sporeforming.
   b. motile and sporeforming.
   c. motile and nonsporeforming.
   d. nonmotile and nonsporeforming.

6. (201) Which of the following genera belongs to the family *Lactobacillaceae?*

   a. *Staphylococcus.*
   b. *Sarcina.*
   c. *Streptococcus.*
   d. *Gaffkya.*

7. (201) A hazy, poorly defined zone of hemolysis that is neither a clear cut alpha nor beta hemolysis can be called

   a. gamma hemolysis.
   b. alpha-prime hemolysis.
   c. nonhemolytic.
   d. "O" type beta hemolysis.

8. (201) Hydrolysis of sodium hippurate by some streptococci yields an end product of

   a. oxidase.
   b. benzoic acid.
   c. ferric chloride.
   d. streptokinase.

9. (201) The clinically important streptococci are classified according to the Lancefield scheme as being in

   a. Group A.
   b. Group B.
   c. Group C.
   d. Group D.

10. (202) The capsule of *Diplococcus pneumoniae* appears to function as a

    a. means of motility.
    b. measure of its Gram-stain ability.
    c. means of producing "R" type colonies.
    d. barrier against phagocytosis.

11. (202) The Neufeld-Quellung reaction is based upon

a. the lysis of colonies using optochin discs.
b. capsular swelling.
c. cellular agglutination.
d. sodium hippurate hydrolysis.

12. (203) The diversity of shapes seen as a result of the distortion of the cell wall of *Corynebacterium diphtheriae* is a result of

a. spores.
b. flagella.
c. Babes-Ernst granules.
d. an exotoxin.

13. (203) To demonstrate in-vitro virulence of *Corynebacterium diphtheriae*, you can use

a. guinea pigs.
b. Klebs-Loeffler (KL) medium.
c. Loeffler's serum agar slants.
d. potassium tellurite agar plates.

14. (204) Which of the following are most likely to be isolated from a patient suffering from "glandular fever"?

a. *Listeria monocytogenes.*
b. *Corynebacterium acnes.*
c. *Corynebacterium hoffmanii.*
d. *Erysipelothrix insidiosa.*

15. (204) Growth of *Erysipelothrix insidiosa* on simple artificial media can be enhanced by the addition of

a. serum or sodium chloride.
b. serum or glucose.
c. glucose or sodium chloride.
d. serum or sucrose.

16. (204) Growth of *Listeria monocytogenes* on sheep blood agar is easily distinguished from beta hemolytic streptococci by

a. a stained slide which shows the difference in morphology.
b. presence of beta hemolysis.
c. colonial morphology.
d. time: 48 hours is required for beta hemolysis to be produced by *Listeria monocytogenes* on sheep blood agar.

17. (205) The capsule of *Bacillus anthracis* differs from the polysaccharide capsule of other bacteria in that the capsule of *Bacillus anthracis*

a. renders it more resistant to autoclaving.
b. is not visible when stained with a capsule stain.
c. is composed of a polypeptide.
d. is not apparently associated with virulence.

18. (205) Although the *Bacillus anthracis* organisms can be demonstrated by preparing smears of blood of infected animals, the most productive demonstration is to examine smears of the

a. bone marrow.
b. spleen.
c. urine sediment.
d. lung.

19. (206) The easiest and most practical way to differentiate members of the genus *Clostridium* from the other Gram-positive bacilli is by

a. checking for hemolysis.
b. determining the spore location.
c. using anaerobic incubation.
d. checking for absence of metachromatic granules.

20. (206) In cases of gangrene, the gangrenous process extends to other body tissues as a result of

a. bacterial motility.
b. endotoxins.
c. the aerobic conditions.
d. exotoxins.

21. (206) The disease known as tetanus (lock jaw) is caused by

a. a true infection with clostridia organisms accompanied by severe and extensive tissue destruction.
b. ingestion of food in which clostridia organisms have produced a highly potent exotoxin.
c. intoxication from exotoxins produced by clostridia organisms growing in wounds.
d. ingestion of food in which heat stable (resistant to boiling) exotoxins have been produced by clostridia organisms.

Chapter 2

22. (207) Opthalmia neonatorium, an eye infection of the newborn, is usually caused by

a. *Neisseria flava.*
b. *Neisseria cabiae.*
c. *Neisseria gonorrhoeae.*
d. *Neisseria meningitidis.*

23. (207) A medium that suppresses growth of many of the saprophytic *Neisseria* spp and yet allows *N. gonorrhoeae* and *N. meningitidis* to grow is

a. Thayer-Martin.
b. supplemented chocolate.
c. Mueller-tellurite.
d. supplemented sheep blood.

24. (207) After 48 hours incubation on chocolate agar, pathogenic *Neisseria* spp. colonies will appear

a. small, rough, grey, and transparent.
b. large, glistening, yellow, and opaque.
c. small, glistening, and translucent.
d. yellow-green surrounded by a whitish area.

25. (207) The oxidase test is used to detect the presence of

a. dimethyl-p-phenylene diamine.
b. cytochrome oxidase.
c. hydrogen peroxide.
d. nonpathogenic *Neisseria* spp.

26. (208) Colonies of Pleuro-pneumonia-like organisms can be distinguished from bacterial colonies because PPLO colonies

a. do *not* decolorize Dienes' strain, but bacterial colonies do decolorize the stain.
b. grow on avian blood agar but *not* on mammalian blood agar.
c. grow only on blood agar that contains 50 percent alcohol.
d. grow only in tissue cultures.

27. (208) Which of the following is considered to be an anaerobe?

a. *Neisseria meningitidis.*
b. *Mima polymorpha.*
c. *Herellea vaginicola.*
d. *Veillonella alcalescens.*

28. (208) *Herellea vaginicola* differs from the other organisms of the tribe Mimeae and the genus *Neisseria* in that

a. it grows well on Thayer-Martin medium.
b. beta type hemolysis may be seen on occasion.
c. it oxidizes xylose.
d. it is peroxidase positive.

29. (208) Special staining procedures are needed for *Mycoplasma* spp. because

a. with Gram stain the flagella stain one color and the cell wall stains another; therefore, the organisms appear Gram-variable.
b. the cell wall contains waxy materials that prevent the penetration of ordinary Gram stain.
c. the capsular material is not soluble in alcohol.
d. the cell wall is poorly defined or absent.

Chapter 3

30. (209) Certain *Hemophilus* organisms cause nonsyphilitic veneral lesions called

a. chancres.
b. chancroids.
c. buboes.
d. veneral warts.

31. (209) Members of the genus *Pasteurella* are

a. microaerophilic.
b. strict aerobes.
c. strict anaerobes.
d. faculative anaerobes.

32. (209) The etiologic agent of plague is

a. *Francisella tularensis.*
b. *Pasteurella pestis.*
c. *Pasteurella multocida.*
d. *Pasteurella pseudotuberculosis.*

33. (210) The three major ingredients in Bordet-Gengou agar are glycerol, potato, and

a. the heat-labile V factor.
b. the heat-stable X factor.
c. 15 percent blood.
d. starch.

34. (210) Members of the genera *Hemophilus* and *Bordetella* are most often found in the

a. nasopharynx.
b. bloodstream.
c. urine.
d. skin lesions.

35. (210) When preparing chocolate agar for use in cultivating *Hemophilus* spp., commercially available enrichment material is added to

a. aid in "chocolating" the red blood cells.
b. replace the heat-labile V factor.
c. replace the heat-stable X factor.
d. maintain a soft, moist agar surface.

36. (210) A term used to describe *Hemophilus* spp. growing in close proximity to a colony of another species is

a. satellitism.
b. isolationism.
c. susceptibility.
d. nonfastidiousness.

37. (210) Which of the following is the etiologic agent of chancroids?

a. *Hemophilus aegyptius.*
b. *Bordetella pertussis.*
c. *Hemophilus influenzae.*
d. *Hemophilus ducrevi.*

38. (211) *Brucella melitensis* causes a disease usually associated with

a. pigs.
b. cows.
c. goats.
d. horses.

39. (212) The *Bacteroides* are normal inhabitants of the mouth, intestinal tract, and vagina. These organisms appear to

a. be the primary cause of purulent pneumonia.
b. be the causative agent of ulcerative colitis.
c. cause asthma in the newborn.
d. be secondary invaders associated with diseased or injured tissue.

40. (211) To differentiate among smooth, rough, and mucoid *Brucella* spp. colonies, you would use

a. a wire loop.
b. thionin and basic fuchsin.
c. an aqueous solution of crystal violet.
d. growth with or without X or V factors.

Chapter 4

41. (213) The IMVIC reaction includes tests for indole, methyl red, Voges-Proskauer, and

a. carbohydrate fermentation.
b. catalase.
c. coagulase.
d. citrate.

42. (213) Which of the following does *not* belong in the family Enterobacteriaceae?

a. *Salmonella* spp.
b. *Alcaligenes* spp.
c. *Shigella* spp.
d. *Escherichia* spp.

43. (213) Which of the following is found in triple sugar iron agar but not found in Kligler's iron agar?

a. Sucrose.
b. Glucose.
c. Lactose.
d. Source of H_2S.

44. (213) Of the organisms we have studied, all enterics

a. are nonspore producers.
b. show a distinct bacillary form.
c. are nonmotile.
d. form capsules.

45. (214) Infections with *Shigella dysenteriae*, serotype 1, are more severe than the other *Shigella* spp. because in addition to producing an endotoxin it also

a. forms capsules.
b. produces a neurotoxin.
c. is a lactose fermenter.
d. produces spores.

46. (214) *Paracolobactrum intermedium* organisms are classified with the

a. *Citrobacter* group.
b. *Proteus-Providence* division.
c. *Shigella* division.
d. *Escherichia coli* organisms.

47. (214) An alkaline reaction on citrate-mannitol agar is evidenced by

a. no color change.
b. a green color.
c. a yellow color.
d. a blue color.

48. (214) Organisms of the *Salmonella-Arizona-Citrobacter* division are separated taxonomically from the *Shigella-Escherichia* division on the basis of

a. the IMViC reaction
b. H_2S production.
c. the IMViC reaction and H_2S production.
d. oxygen and temperature requirements.

49. (215) Which of the following is most likely to yield an acid butt and an acid slant on TSI agar?

a. *Alcaligenes* spp.
b. *Shigella* spp.
c. *Salmonella* spp.
d. *Enterobacter* spp.

50. (215) Serological cross reactions among Proteus, Salmonella, and Escherichia spp. are due to the presence of:

a. lactose fermentation waste products.
b. V antigens.
c. H antigens.
d. O antigens.

51. (216) Cholera causes the patient to have a severe diarrhea known as

a. the GI's.
b. rice water stool.
c. mucus colitis.
d. ulcerative colitis.

52. (216) The disease "melioidosis" in man is caused by

a. *Pseudomonas pseudomallei.*
b. *Alcaligenes fecalis.*
c. *Pseudomonas aeruginosa.*
d. *Citrobacter freundii.*

53. (216) *Vibrio cholerae* is tentatively identified by

a. the presence of the surface pellicle.
b. growth in peptone water.
c. positive cholera-red reaction.
d. its appearance on Gram-stained smears.

Chapter 5

54. (217) Hansen's bacillus is another name for

a. *Mycobacterium tuberculosis.*
b. *Mycobacterium leprae.*
c. *Nocardia asteroides.*
d. *Actinomyces bovis.*

55. (217) Bloodstream dissemination of the tubercle organism can result in

a. Hansen's disease.
b. lettuce seed tuberculosis.
c. tubercular plague.
d. miliary tuberculosis.

56. (217) The mycobacteria that produce a yellow pigmented colony only in the presence of light are placed in Runyon Group

a. I–Photochromogens.
b. II–Scotochromogens.
c. III–Nonphotochromogens.
d. IV–The rapid growers.

57. (218) In order to follow the method of reporting acid-fast bacilli as set up by the National Tuberculosis Association, you would report ten or more acid-fast bacilli per slide as

a. "rare acid-fast bacilli seen."
b. "resubmit specimen."
c. "numerous acid-fast bacilli seen."
d. "few acid-fast bacilli seen."

58. (217) The term "Battey bacilli" refers to members of the

a. rapid growers (Gp. IV).
b. nonphotochromogens (Gp. III).
c. scotochromogens (Gp. II).
d. photochromogens (Gp. I).

59. (218) Which of the media listed below would *least* likely be used to cultivate the tubercle bacilli?

a. Petragnani's.
b. Lowenstein-Jensen.
c. Loeffler's serum slant.
d. Middlebrook 7H10.

60. (219) In indirect tubercule bacilli sensitivity testing, a minimum of how many colonies should be emulsified to make an inoculum?

a. 5.
b. 4.
c. 2.
d. 1.

61. (219) A positive niacin test is indicated by a

a. lack of color change.
b. pink color.
c. blue color.
d. yellow color.

62. (218) The atypical group of mycobacteria can be differentiated from the Runyon groups on the basis of

a. growth at 25° C.
b. colony color.
c. "cauliflower" colonies.
d. growth under anaerobic conditions.

63. (219) A medium containing tripotassium phenolphthalein disulfate would most likely be used to test for

a. catalase.
b. sulfatase.
c. niacin production.
d. antibiotic sensitivity.

Chapter 6

64. (220) In which of the following families will you find the genus that causes the disease "relapsing fever"?

a. Actinomycetaceae.
b. Spirochaetaceae.
c. Achromobacteriaceae.
d. Treponemataceae.

65. (220) Relapsing fever is transmitted from man to man

a. by ticks.
b. through body lice.
c. by the bite of rodents.
d. through bad water.

66. (220) The disease "trench mouth" is caused by *Fusobacterium fusiformis* and

a. *Treponema mucosum.*
b. *Borrelia recurrentis.*
c. *Leptospira canicola.*
d. *Borrelia vincentii.*

67. (220) Laboratory diagnosis of *Borrelia* is made by finding the organism in

a. pus.
b. sputum.
c. the blood.
d. the spinal fluid.

68. (221) From the description of the principles of darkfield microscopsy, you could assume that a very large object would be

a. dark.
b. dim.
c. not visible.
d. very bright.

69. (221) During secondary syphilis, the most logical place to isolate *Treponema pallidum* would be the

a. skin lesions that make up the rash.
b. urethra.
c. chancre.
d. bloodstream.

70. (221) The recommended method for sending fluid from syphilitic lesions to reference laboratories is to collect it

a. in a syringe.
b. in a capillary tube and seal with paraffin-petrolatum.
c. in a small glass tube.
d. on a microscope slide and seal with petrolatum.

71. (221) The most acceptable method for straining treponemes is

a. silver impregnation.
b. nigrosin.
c. India ink.
d. modified acid-fast.

72. (222) Which of the following is the etiologic agent for "Weil's disease"?

a. *Spirochaeta.*
b. *Treponema.*
c. *Leptospira.*
d. *Borrelia.*

73. (221) A lesion that is to be examined for treponemes should *not* be

a. pinched.
b. abraded.
c. washed with saline.
d. washed with germicide.

74. (222) Which of the following has the terminal third of the organism quite flexible and may or may not form a hook?

a. *Treponema* spp.
b. *Leptospira* spp.
c. *Borrelia* spp.
d. *Spirochaeta* spp.

293

90412 02 S01 0370

SUPPLEMENTARY MATERIAL

CDC 90412

MEDICAL LABORATORY TECHNICIAN–MICROBIOLOGY

Volume 2

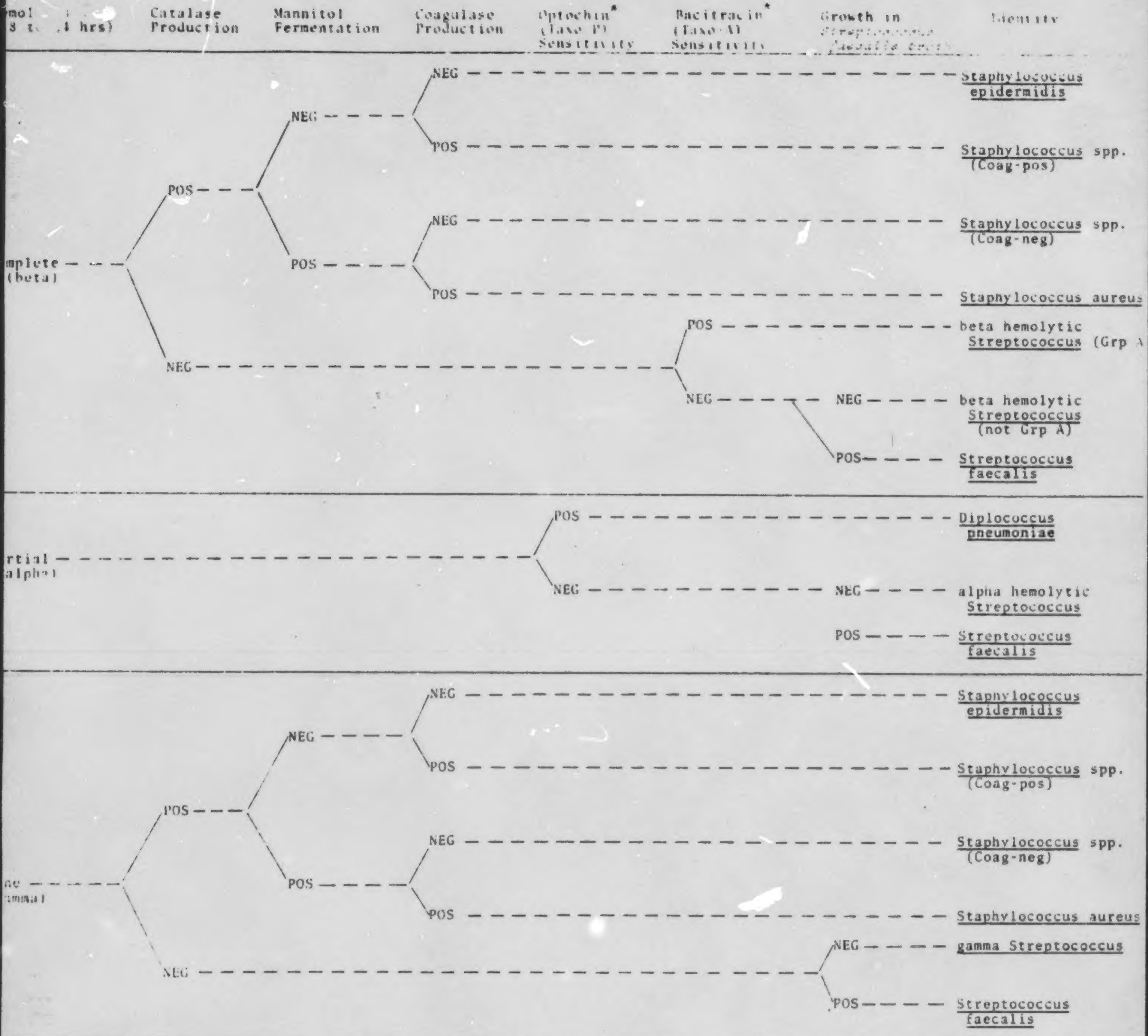
Foldouts 1–8

10-3

Extension Course Institute

Air University

312


mol
3 t .4 hrs)
Catalase Production
Mannitol Fermentation
Coagulase Production
Optochin* (Taxo P) Sensitivity
Bacitracin* (Taxo A) Sensitivity
Growth in Streptococcus faecalis broth
Identity
mplete
(beta)
POS
NEG
NEG
POS
NEG
POS
NEG
POS
POS
NEG
NEG
POS
Staphylococcus epidermidis
Staphylococcus spp. (Coag-pos)
Staphylococcus spp. (Coag-neg)
Staphylococcus aureus
beta hemolytic Streptococcus (Grp A
beta hemolytic Streptococcus (not Grp A)
Streptococcus faecalis
rtial
alpha)
POS
NEG
NEG
POS
Diplococcus pneumoniae
alpha hemolytic Streptococcus
Streptococcus faecalis
ne
amma)
POS
NEG
NEG
POS
NEG
POS
NEG
POS
NEG
Staphylococcus epidermidis
Staphylococcus spp. (Coag-pos)
Staphylococcus spp. (Coag-neg)
Staphylococcus aureus
gamma Streptococcus
Streptococcus faecalis

ootnote: Optochin (Taxo-P)® is commercially prepared ethyl hydrocuprein hydrochloride. Disc diffusion tests will yield a zone of inhibition around colonies of pneumococci. Bacitracin (Taxo-A)® diffusion discs give a zone of inhibition around Group A streptococci.

FOLDOUT 1. IDENTIFICATION OF GRAM-POSITIVE COCCI

Genus	Catalase	Motility	Metachromatic Granules	Oxygen Requirements
Corynebacterium	+	-	Usually present	Aerobic Facultative anaerobe
Listeria	+	+	Usually absent	Aerobic Facultative anaerobe
Erysipelothrix	-	-	Usually absent	Microaerophilic

FOLDOUT 2

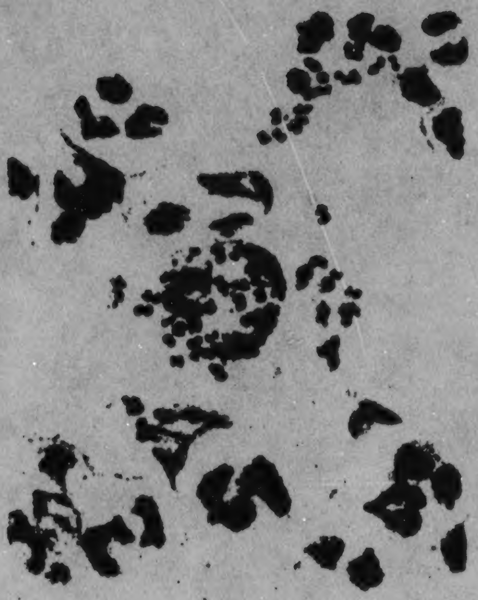
GENERA DIFFERENTIATION WITHIN THE FAMILY CORYNEBACTERIACEAE

ORGANISM	*Hemolysis*	*Spores*	*Indol*	*Nitrate*	*Dextrose*	*Lactose*	*Maltose*	*Salicin*	*Sucrose*	*Gelatin*	*Iron milk*	*Motility*	COMMENTS
Cl. aerofetidum	—	S*	—	+	AG	AG	AG	AG	—	+	ACGD	+	*Spores rarely seen
Cl. bifermentans	+	S	+	—	AG	—	AG	AG	—	+	CDB	+*	*Young cultures only (Urease negative)
Cl. botulinum (Type A-B)	+	S	—	—	AG	—	AG	V	V	+	A	+	
Cl. botulinum (Type C-E)	+	S	—	—	AG	—	AG	V	V	+	CD	+	
Cl. butyricum	—	S	—	—	AG	AG	AG	AG	AG	—	ACG	+	
Cl. chauvoei	+	S	—	+	AG	AG	AG	AG	AG	+	ACG	+	
Cl. fallax	+	S*	—	—	AG	V	AG	V	V	—	ACG	+	*Spores rarely seen
Cl. histolyticum	+	S	—	—	—	—	—	—	—	+	ACD	+	
Cl. multifermentans	+	C,S	—	+	A	A	A	A	A	—	ACG	+	
Cl. novyi (Type A)	+	S	—	—	A	—	V	—	—	+	CG	+	
Cl. novyi (Type B)	+	S	—	—	A	—	V	—	—	+	D	+	
Cl. perfringens	+	C,S	—	+	AG	AG	AG	V	AG	+B	ACG	—	
Cl. septicum	+	S	—	+	AG	AG	AG	AG	—	+	CG	+	
Cl. sordelli	+	S	+	—	AG	—	AG	—	—	+	CDB	+/—	(Urease positive)
Cl. sphenoides	+	S,T	—	—	AG	AG	AG	AG	V	—	AC	+	
Cl. sporogenes	+	S	—	+*	AG	—	AG	—	—	+B	DB	+	*Rapid but no nitrite produced.
Cl. tertium	+	T	—	+	AG	AG	AG	AG	AG	—	ACG	+	Microaerophilic
Cl. tetani	+	T	—	—	—	—	—	—	—	+*	ACG	+	*Blackening
Cl. tetanomorphum	+	T	—	—	AG	—	AG	—	—	+	—	+	

KEY + = positive — = negative A = acid B = blackening C = clot D = digestion G = gas
SPORE C = central T = terminal S = subterminal V = variable

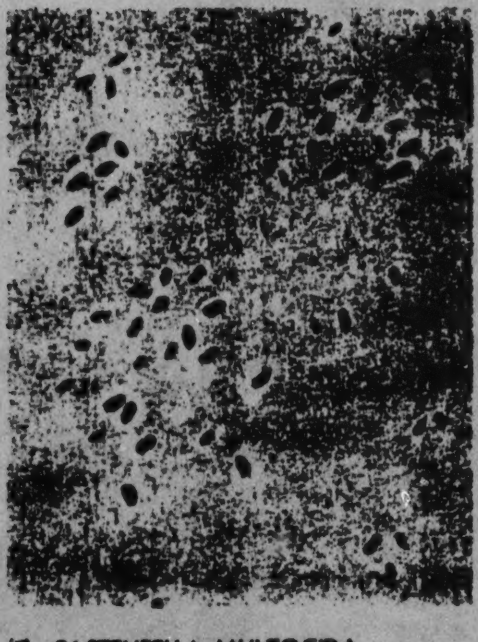
FOLDOUT 3

SPECIES DIFFERENTIATION OF THE CLINICALLY ENCOUNTERED CLOSTRIDIA



(A) NEISSERIA GONORRHOEAE
Gram stain of urethral smear.

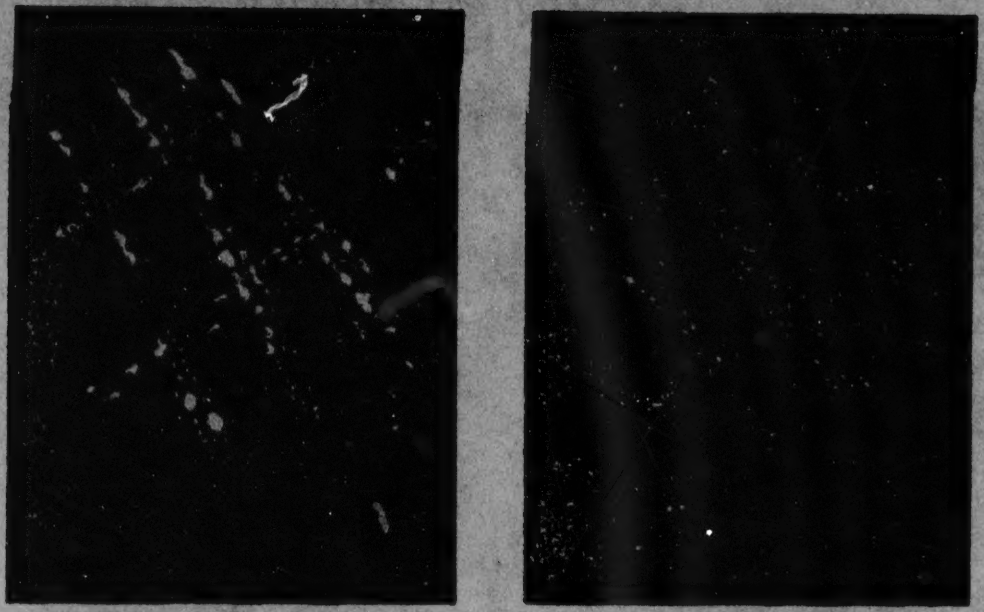
(B) NEISSERIA GONORRHOÉAE
Gram stain of culture smear.



(F) PASTEURELLA MULTOCIDA
Gram stain of culture smear.

(G) HEMOPHILUS INFLUENZAE
Gram stain of culture smear.

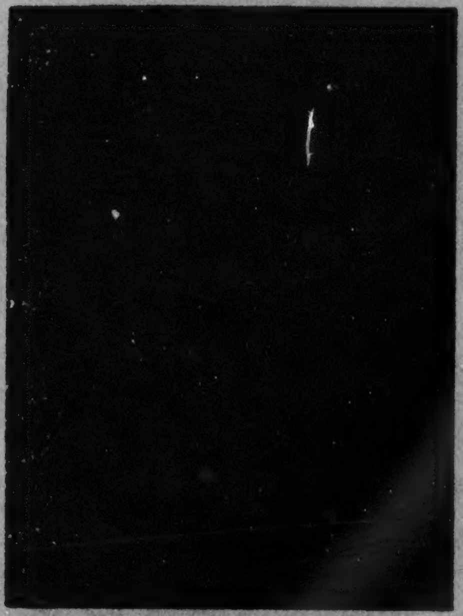
FOLDOUT 4A



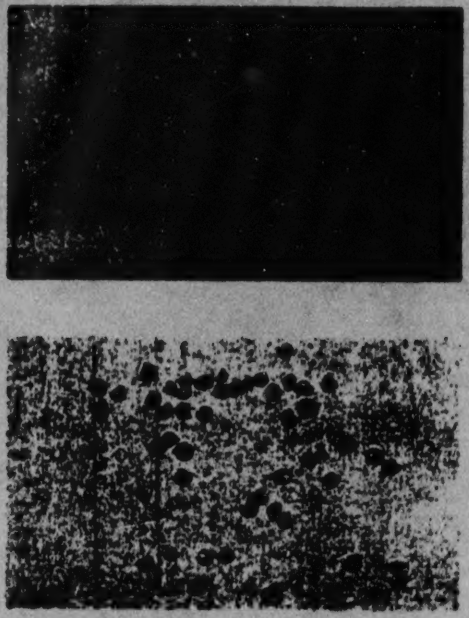
Before Reagent

After Reagent

(E) NEISSERIA GONORRHOEAE - Selection in a mixed culture by oxidase reaction.

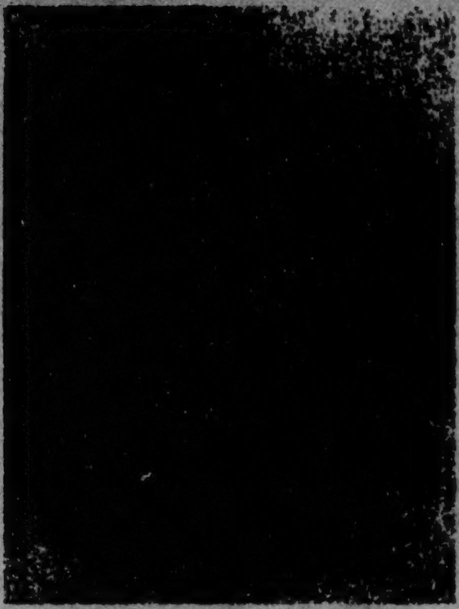


(I) SHIGELLA SPECIES
Culture on EMB agar.

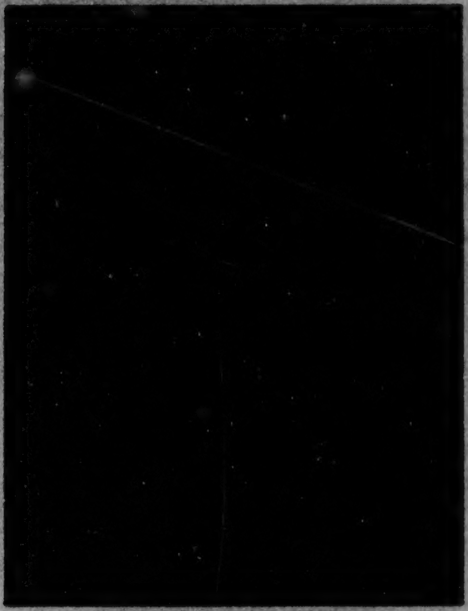


(J, K) SALMONELLA SPECIES
Top: Pure culture on EMB agar.
Bottom: Pure culture on MacConkey's agar.

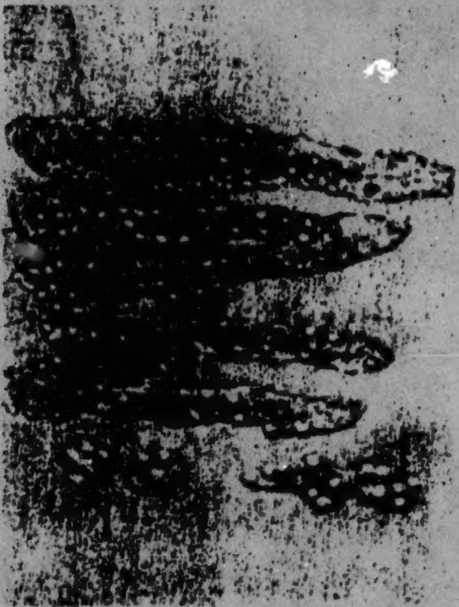
Foldout 4A. Details A through K.



(L) **ESCHERICHIA COLI**
Culture on MacConkey's agar.



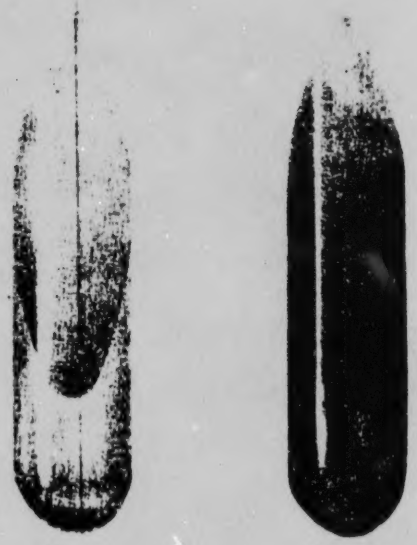
(M) **ESCHERICHIA COLI**
Culture on EMB agar.



(P) **PSEUDOMONAS AERUGINOSA**
Culture on Mueller-Hinton agar.

(Q) **MYCOBACTERIUM TUBERCULOSIS**
Smear of concentrated sputum speciman stained with Ziehl-Neelsen acid-fast stain.

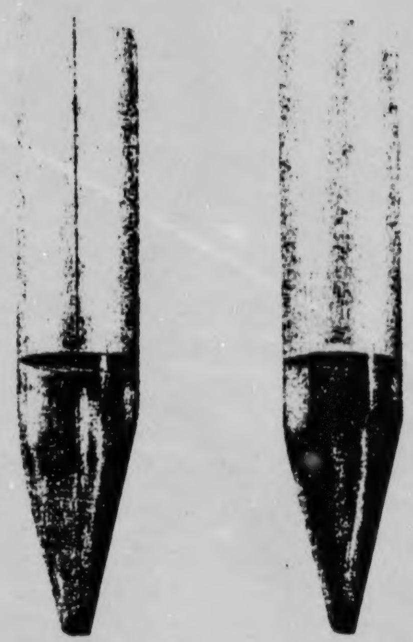
FOLDOUT 4B



(O) PROTEUS SPECIES
Urease production.
Left: Negative reaction of E. coli.
Right: Positive reaction.

(T) MYCOBACTERIUM

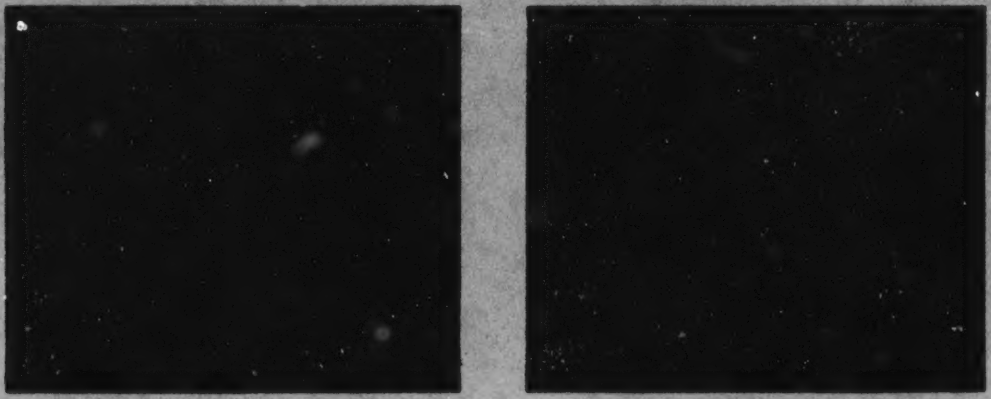
Photochromogen on Lowenstein-Jensen medium. Left: growth for five days in the dark at room temperature. Right: yellow pigment by incubation in sunlight.



(U) MYCOBACTERIUM TUBERCULOSIS

Niacin production. Left: Negative (colorless) Right: Positive test (lemon yellow color).

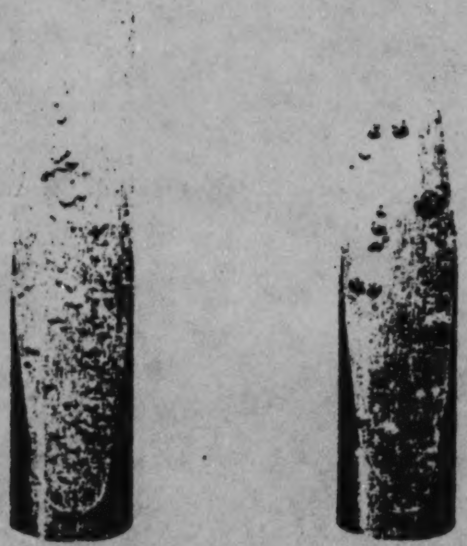
Foldout 48. Details L through U.



Discrete Colonies

Swarming Colonies

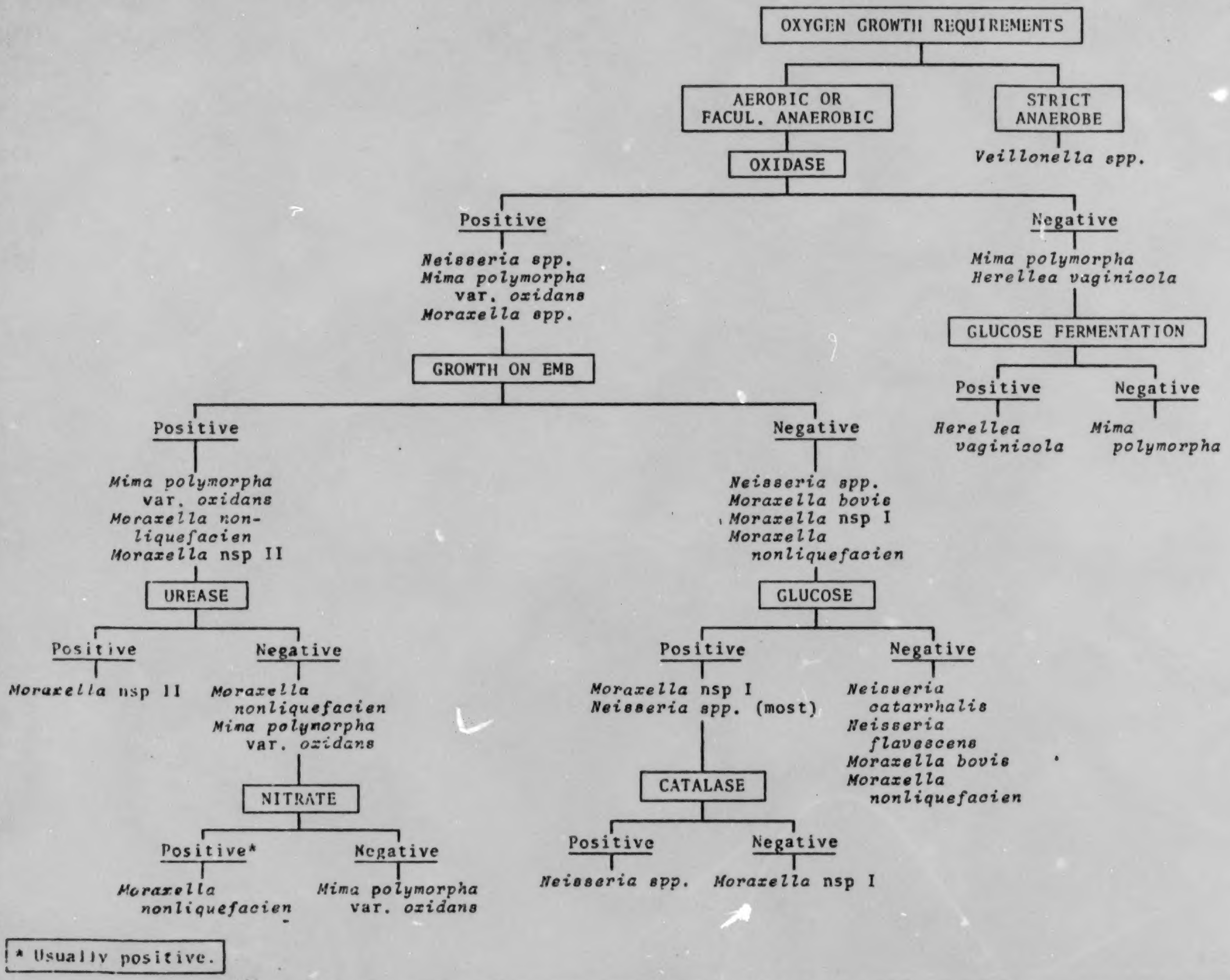
(N) PROTEUS SPECIES - On blood agar.



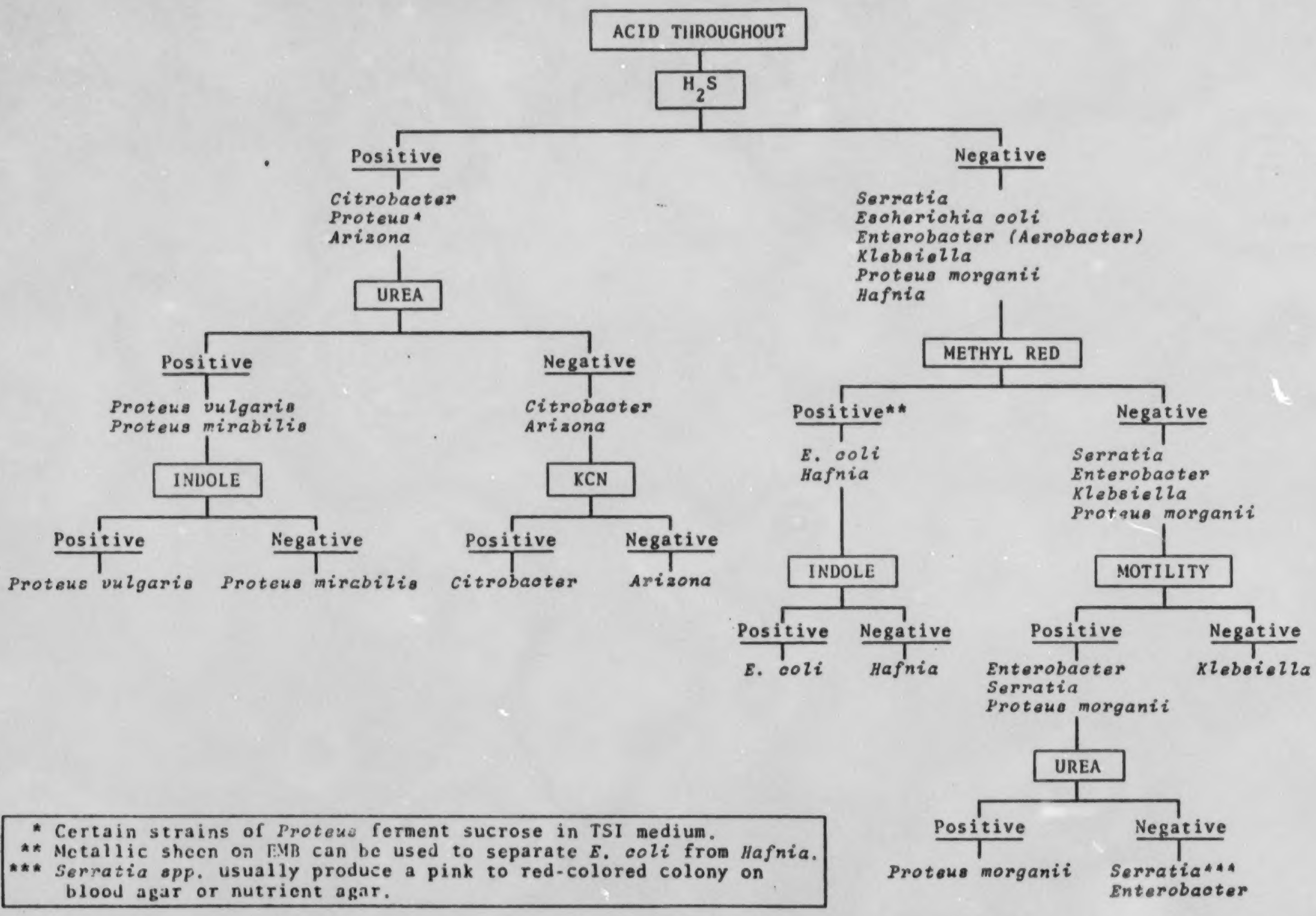
(R) MYCOBACTERIUM TUBERCULOSIS
Human type. Cultures of concentrated sputum specimen on Lowenstein-Jensen medium.

(S) MYCOBACTERIUM
Atypical or anonymous type. Scotochromogen on Lowenstein-Jensen medium. Yellow-orange pigment is formed even in darkness.

FOLDOUT 4B

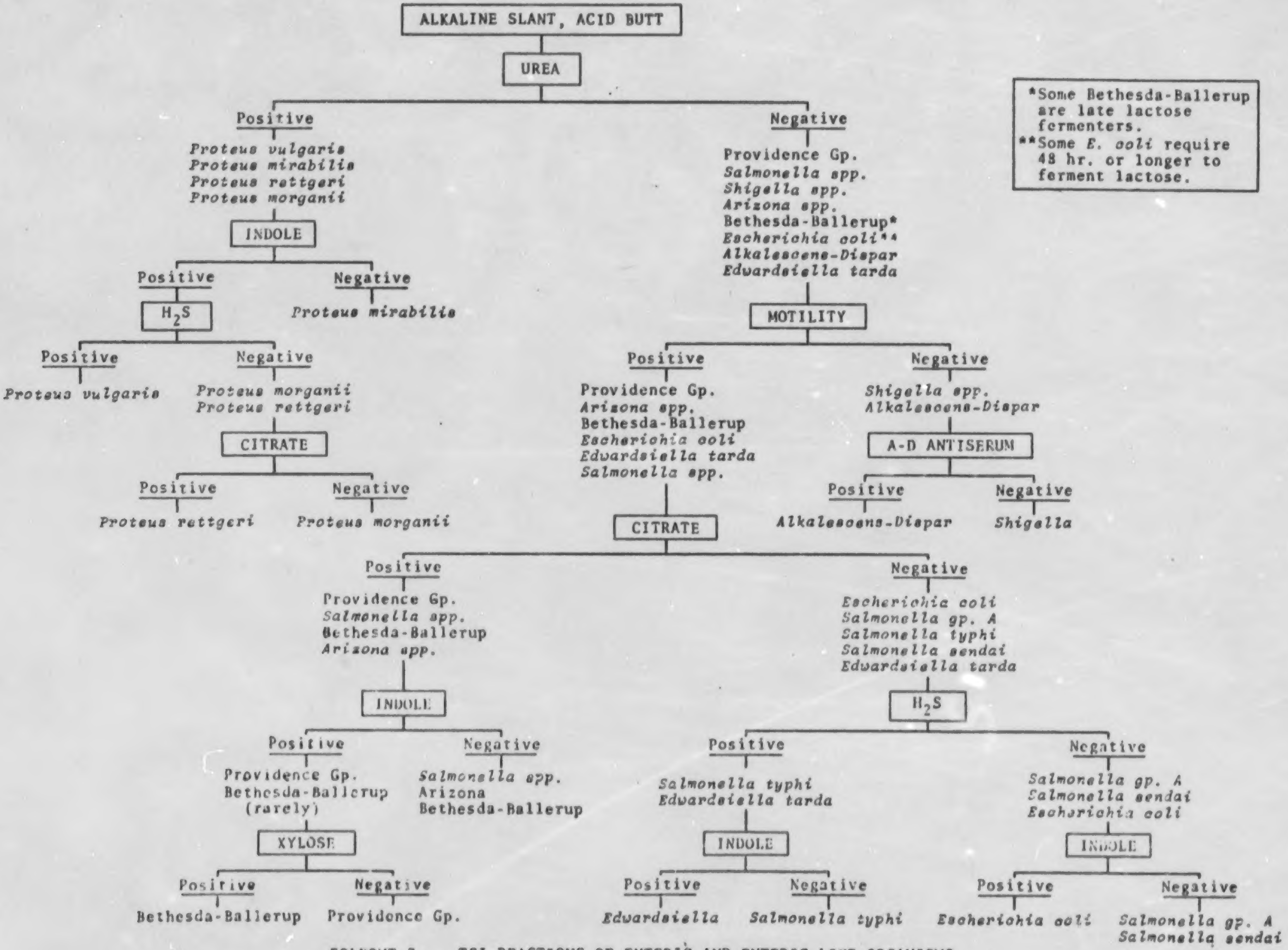

OXYGEN GROWTH REQUIREMENTS
AEROBIC OR FACUL. ANAEROBIC
STRICT ANAEROBE
Veillonella spp.
OXIDASE
Positive
Neisseria spp.
Mima polymorpha var. *oxidans*
Moraxella spp.
Negative
Mima polymorpha
Herellea vaginicola
GLUCOSE FERMENTATION
Positive
Herellea vaginicola
Negative
Mima polymorpha
GROWTH ON EMB
Positive
Mima polymorpha var. *oxidans*
Moraxella nonliquefacien
Moraxella nsp II
Negative
Neisseria spp.
Moraxella bovis
Moraxella nsp I
Moraxella nonliquefacien
UREASE
Positive
Moraxella nsp II
Negative
Moraxella nonliquefacien
Mima polymorpha var. *oxidans*
NITRATE
Positive*
Moraxella nonliquefacien
Negative
Mima polymorpha var. *oxidans*
GLUCOSE
Positive
Moraxella nsp I
Neisseria spp. (most)
Negative
Neisseria catarrhalis
Neisseria flavescens
Moraxella bovis
Moraxella nonliquefacien
CATALASE
Positive
Neisseria spp.
Negative
Moraxella nsp I
* Usually positive.

FOLDOUT 5. DIFFERENTIATION OF GRAM-NEGATIVE DIPLOCOCCI AND COCCOID ORGANISMS


ACID THROUGHOUT
H_2S
Positive
Citrobacter
Proteus*
Arizona
UREA
Positive
Proteus vulgaris
Proteus mirabilis
INDOLE
Positive
Proteus vulgaris
Negative
Proteus mirabilis
Negative
Citrobacter
Arizona
KCN
Positive
Citrobacter
Negative
Arizona
Negative
Serratia
Escherichia coli
Enterobacter (Aerobacter)
Klebsiella
Proteus morganii
Hafnia
METHYL RED
Positive**
E. coli
Hafnia
INDOLE
Positive
E. coli
Negative
Hafnia
Negative
Serratia
Enterobacter
Klebsiella
Proteus morganii
MOTILITY
Positive
Enterobacter
Serratia
Proteus morganii
UREA
Positive
Proteus morganii
Negative
Serratia***
Enterobacter
Negative
Klebsiella

* Certain strains of *Proteus* ferment sucrose in TSI medium.
** Metallic sheen on EMB can be used to separate *E. coli* from *Hafnia*.
*** *Serratia spp.* usually produce a pink to red-colored colony on blood agar or nutrient agar.

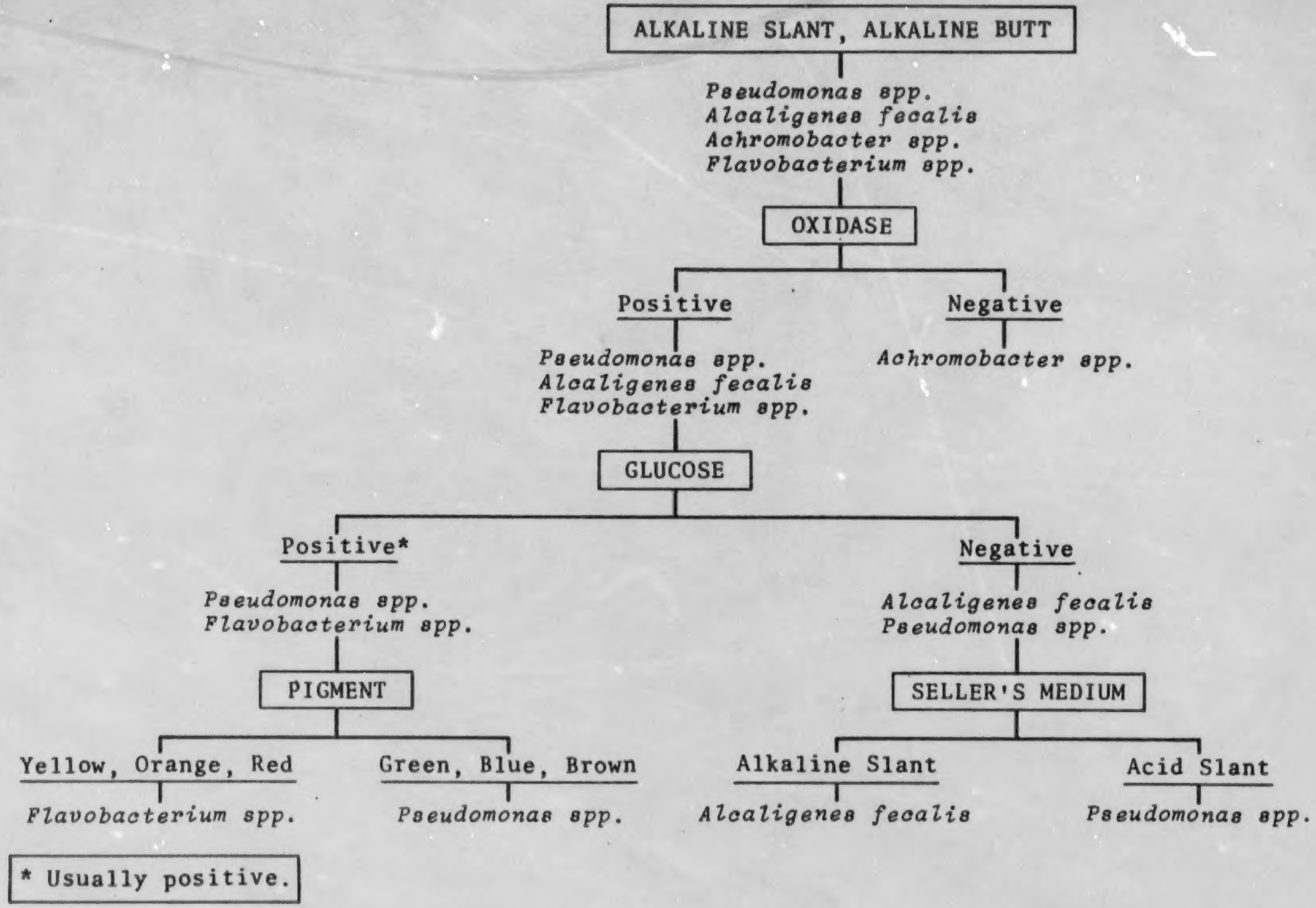
FOLDOUT 6. TSI REACTIONS OF ENTERIC AND ENTERIC-LIKE ORGANISMS


ALKALINE SLANT, ACID BUTT
UREA
Positive
Proteus vulgaris
Proteus mirabilis
Proteus rettgeri
Proteus morganii
INDOLE
Positive
H_2S
Positive
Proteus vulgaris
Negative
Proteus morganii
Proteus rettgeri
CITRATE
Positive
Proteus rettgeri
Negative
Proteus morganii
Negative
Proteus mirabilis
Negative
Providence Gp.
Salmonella spp.
Shigella spp.
Arizona spp.
Bethesda-Ballerup*
Escherichia coli**
Alkalescens-Dispar
Edwardsiella tarda
MOTILITY
Positive
Providence Gp.
Arizona spp.
Bethesda-Ballerup
Escherichia coli
Edwardsiella tarda
Salmonella spp.
CITRATE
Positive
Providence Gp.
Salmonella spp.
Bethesda-Ballerup
Arizona spp.
INDOLE
Positive
Providence Gp.
Bethesda-Ballerup
(rarely)
XYLOSE
Positive
Bethesda-Ballerup
Negative
Providence Gp.
Negative
Salmonella spp.
Arizona
Bethesda-Ballerup
Negative
Escherichia coli
Salmonella gp. A
Salmonella typhi
Salmonella sendai
Edwardsiella tarda
H_2S
Positive
Salmonella typhi
Edwardsiella tarda
INDOLE
Positive
Edwardsiella
Negative
Salmonella typhi
Negative
Salmonella gp. A
Salmonella sendai
Escherichia coli
INDOLE
Positive
Escherichia coli
Negative
Salmonella gp. A
Salmonella sendai
Negative
Shigella spp.
Alkalescens-Dispar
A-D ANTISERUM
Positive
Alkalescens-Dispar
Negative
Shigella

*Some Bethesda-Ballerup are late lactose fermenters.

**Some *E. coli* require 48 hr. or longer to ferment lactose.

FOLDOUT 7. TSI REACTIONS OF ENTERIC AND ENTERIC-LIKE ORGANISMS


ALKALINE SLANT, ALKALINE BUTT
Pseudomonas spp.
Alcaligenes fecalis
Achromobacter spp.
Flavobacterium spp.
OXIDASE
Positive
Pseudomonas spp.
Alcaligenes fecalis
Flavobacterium spp.
Negative
Achromobacter spp.
GLUCOSE
Positive*
Pseudomonas spp.
Flavobacterium spp.
PIGMENT
Yellow, Orange, Red
Flavobacterium spp.
Green, Blue, Brown
Pseudomonas spp.
Negative
Alcaligenes fecalis
Pseudomonas spp.
SELLER'S MEDIUM
Alkaline Slant
Alcaligenes fecalis
Acid Slant
Pseudomonas spp.
* Usually positive.

FOLDOUT 8. TSI REACTIONS OF ENTERIC AND ENTERIC-LIKE ORGANISMS

90412 03 1170 0973

CDC 90412

MEDICAL LABORATORY TECHNICIAN–MICROBIOLOGY

(AFSC 90470)

Volume 3

Clinical Parasitology

10-3

Extension Course Institute
Air University

Preface

THIS IS THE third of four volumes of CDC 90412, Medical Laboratory Technician-Microbiology. It presents information on parasites of medical importance including both Protozoa and Helminths. You will find color illustrations on foldouts, at the back of the volume, that will assist you in learning to identify malaria parasites.

Bound in the back of the volume are three oversized figures printed as Foldouts 1 through 3.

If you have questions on the accuracy or currency of the subject matter of this text or recommendations for its improvement, send them to Technical Training Center (MSSTW), Sheppard AFB, Texas 76311.

If you have questions on course enrollment or administration, or on any of ECI's instructional aids (Your Key to Career Development, Study Reference Guides, Chapter Review Exercises, Volume Review Exercise, and Course Examination), consult your education officer, training officer, or NCO, as appropriate. If he can't answer your questions, send them to ECI, Gunter AFB, Alabama 36114, preferably on ECI Form 17, Student Request for Assistance.

This volume is valued at 30 hours (10 points).

Material in this volume is technically accurate, adequate, and current as of July 1970.

309

Contents

Page

Preface iii

Chapter

1 INTRODUCTION TO MEDICAL PARASITOLOGY 1

2 PROTOZOA INFECTING MAN 6

3 PLATYHELMINTHES INFECTING MAN 25

4 NEMATODES INFECTING MAN 40

333

CHAPTER 1

Introduction to Medical Parasitology

FROM THE EARLIEST recorded history, man has been subjected to animal parasites. The Israelites bewailed their torment by the "fiery serpents" of Egypt during their sojourn by the Red Sea. Could these tormentors have been the guinea worm, *Dracunculus medinensis?* Is it possible that the porkworm *(Trichinella spiralis)* or the pork tapeworm *(Taenia solium)* may have influenced the ancient Jewish elders to condemn pork as unclean? Our knowledge of medical parasitology has increased tremendously in recent years. Yet, the same parasites that have afflicted man throughout history are still the number one cause of disease in much of the world.

2. It is estimated that there are almost fifty million guinea worm infections in the world at the present time. The highest incidence of porkworm was in the United States (approximately 15–20 percent). Strict control measures in the past 20 years have reduced that figure to less than five percent. Servicemen returning from overseas operations are constant reminders that parasites alien to our environment are still flourishing in tropical countries. As a laboratory technician, you have an excellent opportunity to study many of the parasites that continue to plague the human race.

3. This volume is written to provide you with background and technical knowledge associated with the parasites of major medical importance. Its purpose is to establish a foundation with which you can increase your ability and skill in finding, identifying, and discussing human parasites in future laboratory performance. This ability and skill require individual initiative, technical knowledge, and experience.

4. This chapter begins with a discussion of the development of medical parasitology and the military aspects of the subject, followed by classification (nomenclature and identification). General relationships of the host and parasite, with descriptions of how parasites affect the host, are explained next. The chapter ends by presenting information of the four important sources or modes by which parasites are transmitted.

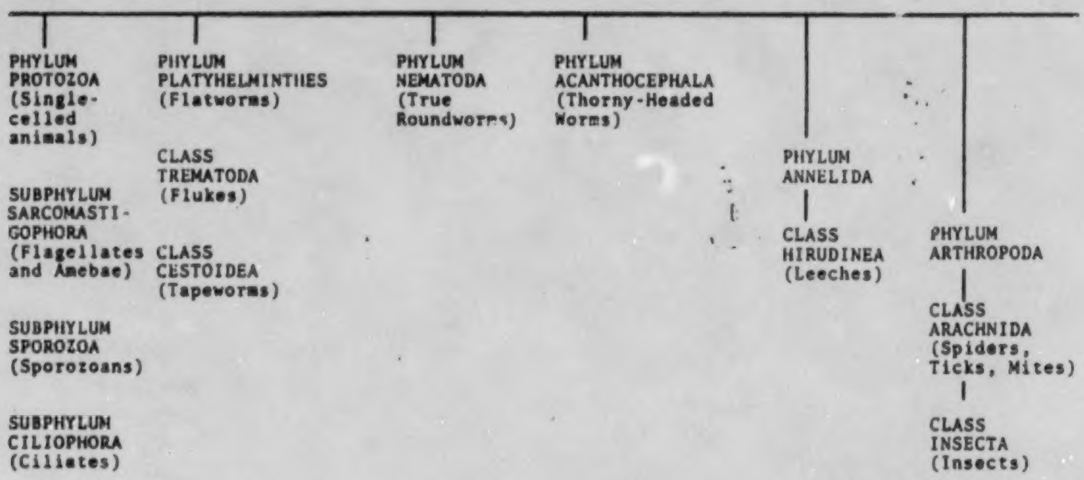
1. Medical Parasitology

1-1. A parasite is any plant or animal which lives on or within another organism, deriving its sustenance or protection without making compensation. The uncompensated organism is referred to as the host. Parasitology is the science of parasitism and deals with the relationship between a parasite and its host. Medical parasitology refers to parasites which infect man. Medical laboratory parasitology is an essential clinical aid which enables a physician to diagnose and treat patients suffering from parasitic infections.

1-2. Macroscopic parasites, such as worms, leeches, maggots, and bedbugs, were known in antiquity. Even today, the alchemy and misconceptions of the ancients are practiced in the darker recess of the world. Village medicine men continue to wind a "fiery serpent" from the flesh of its victim on a crude stick. The science of parasitology has advanced but the advancements are not applied worldwide. With the discovery of the microscope by Van Leeuwenhoek in the late 17th century, a new era in parasitology had its beginning. However, parasitology could not have advanced without concurrent advances in the related fields of protozoology (one-celled animals) and entomology (study of insects). The major developments in modern parasitology came in the 19th century with tremendous technical and scientific efforts in all phases of parasitology and its related fields. The discovery of the human malaria parasite in 1880, closely followed in 1900 by the conclusive demonstration that malaria is contacted through the "bites" of infected mosquitoes, is an example of the accelerated progress in parasitology.

1-3. **Military Medical Parasitology.** Progress in the medical aspects of parasitology was accelerated by necessity during the world conflicts of the 20th century. In World War II (WW II), thousands of Americans were transported to foreign countries. This exposed them to exotic parasitic diseases. Formerly, these maladies were seldom

TABLE 1
PRINCIPAL CLASSES AND PHYLA OF PARASITES OF MEDICAL IMPORTANCE


PHYLUM PROTOZOA (Single-celled animals)
SUBPHYLUM SARCOMASTIGOPHORA (Flagellates and Amebae)
SUBPHYLUM SPOROZOA (Sporozoans)
SUBPHYLUM CILIOPHORA (Ciliates)
PHYLUM PLATYHELMINTHES (Flatworms)
CLASS TREMATODA (Flukes)
CLASS CESTOIDEA (Tapeworms)
PHYLUM NEMATODA (True Roundworms)
PHYLUM ACANTHOCEPHALA (Thorny-Headed Worms)
PHYLUM ANNELIDA
CLASS HIRUDINEA (Leeches)
PHYLUM ARTHROPODA
CLASS ARACHNIDA (Spiders, Ticks, Mites)
CLASS INSECTA (Insects)

encountered in the United States. Amebiasis and opisthorchiasis in Southeast Asia are two examples. Malaria was a constant threat in various parts of the world during WW II. Military medical reports indicate that many more men were incapacitated from disease than from enemy action. With an increased interest in parasitology, techniques were improved. Some of the advancements in diagnosis and treatment of parasitic diseases were gained through the medical experience of World War II.

1-4. During the Korean conflict prophylactic measures were initiated, using the procedures of WW II. Certain strains of malaria subsequently showed increased resistance to prophylactic drugs. As a result, some servicemen contracted malaria despite prophylaxis. Because of knowledge gained in WW II, the incidence of parasitic diseases in general was comparatively low in the Korean conflict, the only exception being malaria.

1-5. In the Vietnam war, malaria again presented a problem. One species of malaria, namely *Plasmodium falciparum*, has developed an even greater resistance to the prophylactic drugs routinely used. It was reported that during 1965 as many men were evacuated from Vietnam because of malaria alone as were evacuated for wounds. The incidence of other parasites among the military population is a less serious problem. This can be seen in a survey of military medical problems in Vietnam that found that of every 1,000 men, 2 had hookworm and 5 had amebiasis.

1-6. Because of the United States' commitments around the world, American servicemen are likely to be assigned to many different areas. As a laboratory technician, you may find yourself in parasite endemic areas where an important part of your laboratory work will be in parasitology. On the other hand, returning servicemen may be assigned to any base within the continental United States. This means that any laboratory technician in the United States may be confronted with a variety of unusual parasites for identification. You may frequently find parasites which are alien to your particular locale. Your identification and the subsequent treatment of diseased patients are necessary for two important reasons. The first involves the mission of a military hospital to return the patient to duty with a minimum loss of man-hours. The second reason is to prevent the introduction and/or distribution of parasites within the United States.

1-7. Medical parasitology can be categorized on the basis of specimens examined (feces, urine, blood, etc.). The source of a specimen is important from the technical aspect of processing it for parasites. The organization of a clinical laboratory into sections for specific specimen handling is conducive to improvement in technician performance. For instance, it is logical to process smears for blood flagellates in the hematology section and stool specimens in the parasitology section. This customary procedure also points out the necessity for technicians in other sections of the laboratory to maintain their proficiency in medical parasitology.

1-8. The parasitology section usually is a separate unit, because of the nature of the primary specimen, feces. As you will see in later chapters, this examination is only a part of medical parasitology. However, fecal examinations are the major workload in a parasitology section. This is mainly because of public health requirements for examination of civilian and military food handlers. In less developed areas of the world, civilian food handlers constitute a serious and continuing health problem in the military population. Thus, a qualified parasitology technician is an essential member of the military public health team.

1-9. **Classification of Parasites.** Before proceeding further, remember that the classification of parasites is established by the International Code of Zoological Nomenclature. Several changes have been approved in the classification of parasites as compared to the nomenclature used only a few years ago.

1-10. Table 1 shows the principal phyla and classes of the parasites of medical importance as they are now classified. For parasites to be placed into a phylum they must have something in common. The fact that there may be more than one class in a phylum denotes that parasites have enough in common to be placed in a particular phylum, but they differ enough to be placed in separate classes.

1-11. Additional charts are provided to demonstrate the grouping of parasites by phylum as they are discussed. The material for the volume as a whole has been selected and organized to provide you with a clear and practical understanding of the key points you must know for the successful identification of human parasites.

2. Host-Parasite Relationships

2-1. Parasites have specific relationships with the animals they infest or infect. These relationships are determined by specific requirements such as a need for food or protection or as an essential step in the life cycle of the parasite. Some parasites infect a wide variety of hosts to which they have become adapted. A few, such as *Enterobius vermicularis*, the human pinworm, only infect one host. As you can see, parasitism is not a haphazard phenomena. Perhaps a closer look at host-parasite relationships will make this subject clearer.

2-2. **Types of Parasites.** Some parasites adapt themselves to the outer coverings of other animals. Thus, we find parasites that live on the skin and in the hair, or they may burrow just beneath the skin. Other parasites enter the body of the organism that they infect. Malaria is a good example here. The malaria parasite enters the body and infects red blood cells. The location within or upon the body where parasites may be found provides a basis for classifying them into two broad categories. These categories are ectoparasites and endoparasites.

2-3. *Ectoparasites*. Those parasites which are found on or within the skin or outside the body are called ectoparasites. These afflictions are called infestations rather than infections. Arthropods such as body lice and ticks are of this type; so are mites which burrow into the skin. Arthropods are the most frequent extoparasites encountered. Our discussion of the arthropods will relate mainly to their role as vectors of parasitic diseases. The identification of ectoparasites is more properly the domain of entomologists.

2-4. *Endoparasites*. Those parasites which are located within the host's body are called endoparasites. These may be found in such areas as the intestines, liver, lungs, blood vessels, and muscles. The majority of medically important parasites are endoparasites. Medical problems related to them are termed infections.

2-5. **The Host.** Let us now consider the host itself. A host, you should recall, is that organism which furnishes food or protection for a parasite. During its life cycle, a parasite may require one or more hosts, or at some stages it may not require a host. In some cases, man as a host appears to be a dead end. An example is *Trichinella spiralis* infection, in which the parasite is walled off in human tissue and cannot be transmitted to another host.

2-6. *Definitive hosts*. A definitive host is one in which a parasite reaches sexual maturity and is able to complete its reproductive functions. In most cases of parasitic infections of man, man is the definitive host. It is at sexual maturity that the parasite is capable of reproducing its own kind and, in so doing, produce evidence of its presence as ova, cysts, proglottids, etc. Patent infections are those which are producing such evidence of their presence.

2-7. *Intermediate hosts*. An intermediate host is one in which a parasite undergoes essential immature development stages. Some parasites, such as the schistosomes, are capable of completing their preliminary development in one intermediate host, a snail. Other parasites, such as *Clonorchis sinensis* (also called *Opisthorchis sinensis)*, may require two intermediate hosts, such as snails and then fresh water fish, in order to complete their preliminary development.

2-8. Man is usually the definitive host. However, man can also be an intermediate host. For example, sexual maturity of the malarial parasite takes place in the mosquito; thus, the mosquito is the definitive host. It is the asexual stage of the malarial parasite which is found in man; thus, man is the intermediate host in this life cycle. Man serves as both the definitive and intermediate host in the case of *Trichinella spiralis*. The female trichinae gives birth to larvae which encyst as the infective stage in the same host.

2-9. The terms "definitive" and "intermediate" host should be used with some reservations. The genus *Leishmania*, for example, has no known means of sexual reproduction. The terms "definitive" and "intermediate" are not applicable in this life cycle.

2-10. *Reservoir hosts.* In addition to the designation "definitive" or "intermediate," a host may also be called a *reservoir* host. A reservoir host is an animal species on which the parasite depends for its survival in nature and thus serves as a source of infection for other susceptible hosts, including man. For example, the hog harbors the organism *Trichinella spiralis,* which causes trichinosis in man.

2-11. *Vectors.* In addition to their role as reservoirs, some hosts serve as vectors. A *vector* is a parasite carrier, usually an arthropod, which transfers parasites from one host to another. This is true of organisms such as mosquitoes, flies, ticks, and mites. Because of the many different kinds of organisms in the world, there are many different types of vectors.

2-12. *Symbiosis.* A close association of two species of organisms in nature is called *symbiosis.* The partners in this association are termed symbionts. This relationship may have several aspects. One aspect is the association in which both organisms benefit, or *mutualism.* A second aspect is an association in which one organism benefits and the other is unaffected. This is known as *commensalism.* The third aspect of symbiosis is *parasitism.* In parasitism, one organism (parasite) is benefited at the expense of the other organism (host). A parasite is successful only when it is in a delicate balance with its host. If that balance is upset, the host may expel or even destroy the parasite, or the host may be damaged to such an extent that it dies and, as a result, the parasite dies. Parasitology is important to us because this balance is not always maintained.

2-13. **Parasitic Effects.** Throughout this volume we will use the terms "definitive," "intermediate," and "reservoir" when referring to a host. Now that we know a little about parasites and hosts, let's discuss the effects of parasites on the host. By definition, each parasite must have at least one host and, as mentioned previously, may have several. For a given parasite to live successfully within a host, several conditions are necessary.

- There must be a dependable means of transmission to the host.
- Environmental conditions must provide suitable access to the host.
- The parasite must be able to thrive and reproduce in the host once access is made.

If any one of these three circumstances is altered or eliminated, a parasite cannot exist. However, if these prerequisites are met, a parasite will be successful.

2-14. Damage to the host can manifest itself in several ways. These are:

- Trauma.
- Lytic action.
- Tissue response.
- Blood loss.
- Secondary infection.

2-15. Traumatic damage to tissue can occur as a result of normal larval migration. For example, in hookworm infections, as the individual larvae penetrate into the air sacs, minute focal hemorrhages are produced. On the other hand, in cases of simultaneous massive migration of larvae, a bronchial pneumonitis of clinical grade is usually produced. Intestinal parasites such as *Ascaris lumbricoides* and *Taenia saginata* may cause intestinal obstruction if the worm population is high. Other parasites may cause trauma to the intestines in their attempt to maintain attachment on the intestinal mucosa. A few parasites are capable of producing damage to the retinal, renal, or cerebral capillaries.

2-16. Lytic destruction or damage can occur by the invasion of certain parasites because of their enzyme activity. The protozoan *Entamoeba histolytica* can colonize in the large intestine, thus causing considerable damage as the organisms invade the tissues by lytic digestion.

2-17. Some parasites, notably helminths (worms), by their invasion of tissue, cause the human host to respond by producing eosinophilia. This production of eosinophiles in the bloodstream is most notable in infections with *Strongyloides stercoralis, Ascaris lumbricoides,* and *Trichinella spiralis.*

2-18. Parasites such as *Ancylostoma duodenale* and *Necator americanus,* when present in large numbers, can cause considerable blood loss. With this loss of red blood cells, the body responds with increased erythropoietic activity. If the body response is not sufficient, an anemia results.

2-19. Once the body becomes weakened by anemia or tissue damage—or both anemia and tissue damage—secondary invaders, usually bacteria, may add to the invasive process. Secondary infection may occur in the liver, lungs, or intestinal mucosa.

2-20. Parasitic infections, unlike bacterial infections, usually produce only short-lived immunity, if any. As a result, repeated infections are quite possible. It is known that long-lasting immunity is derived from infection and spontaneous cure in cutaneous leishmaniasis. However, immunological defenses against parasitic infections are limited.

2-21. **Modes of Infection.** Man becomes infected with parasites by various means. Parasitic diseases may be grouped according to mode of transmission as follows:

- Filth-borne or contaminative.
- Soil- or water-borne.
- Food-borne.
- Arthropod-borne.

2-22. Transmission by filth or contamination is a serious problem where personal hygiene is lacking. Some of the intestinal protozoa are dependent upon this condition for direct transmission. Parasites such as *Dientamoeba fragilis* and *Trichomonas hominis* have no known cystic stage so they must be transmitted directly. Other intestinal protozoa and some intestinal helminths such as *Hymenolepis nana* and *Enterobius vermicularis* are not dependent upon direct transmission, but they are readily transmitted by that route. These organisms produce cysts or eggs that are fully developed when passed and can survive and remain infective for long periods outside the host. As you can see, these parasites can be spread easily by careless food handlers, nursery attendants, etc. Even flies and cockroaches can carry the cysts and eggs from one place to another. Personal hygiene and community sanitation are a must to prevent the spread of these parasites.

2-23. Soil- or water-borne parasitic infections are among the most difficult to control. The eggs of parasites such as *Ascaris, Trichuris,* and hookworms require a period of development in the soil before they become infective. People acquire *Ascaris* and *Trichuris* infections by eating dirt. Of course, this is one of the favorite pastimes of children. Infective hookworm larvae normally infect man by penetrating the skin and, as you would expect, the parasite is most common in areas where people do not wear shoes. The intermediate hosts and the infective stage of blood flukes require water. The cercariae of blood flukes infect man by penetrating the skin when he is exposed to contaminated water (swimming, bathing, etc.). Education of the population and strict sanitary control of excreta disposal are the only effective means of preventing infection with these parasites.

2-24. Man acquires parasitic infections by eating many things other than soil. Inadequately cooked beef, pork, fish, and shellfishes, as well as some vegetables, are important sources of infection. The infective stages of some tapeworms are found in beef, pork, and fish. Eating improperly prepared pork is the most common source of *Trichinella spiralis* in man. Raw crabs and crayfish are considered a delicacy in some parts of the world. They are intermediate hosts that harbor the infective stage of the lung fluke. Some flukes, such as sheep liver-fluke, infect man when he consumes raw aquatic plants. The infective larvae of these flukes encyst on such vegetation. You can see from these examples that food is an important source of infection. To prevent food contamination, excreta must be disposed of according to strict public health rules; and all foods must be properly prepared if these parasites are to be controlled.

2-25. Parasites that are transmitted by arthropod vectors produce diseases that are frequently fatal. Members of this group and their vectors are found in most temperate and tropical regions of the world. Some of these parasites have been significant deterrents to the economic development of some countries. These arthropod-borne parasites include both protozoa and helminths. In each case, the arthropod is an essential intermediate host and vector. Malaria is the most widespread of these parasites, and species of the *Anopheles* mosquito which serve as the vector are found in almost every country of the world. This is the reason that such a great effort is made to prevent infected individuals from entering this country. Some parts of Africa are literally ravaged by sleeping sickness. This often fatal disease is caused by African trypanosomes which are transmitted by tsetse flies. The group of helminths that are arthropod-borne are known as filariids. There are several of these parasites, and each causes a characteristic disease (elephantiasis, ophthalmitis, "Calabar swelling", etc.). The vectors for these parasites include mosquitoes, mango flies, *Simulium* flies, and some biting gnats. Tremendous amounts of money and time have been spent in an effort to eradicate these diseases, but they are still the most important public health problems in the tropics today.

CHAPTER 2

Protozoa Infecting Man

THE SUBPHYLA you will study in this chapter are listed in table 2. The listed subphyla contain the protozoa of major medical importance. These parasites live in the digestive tract, genitalia, tissues, and bloodstream. The majority of the parasites that infect the tissues and bloodstream require a vector. Most of these protozoa are pathogenic, but a few are nonpathogenic or only cause mild tissue irritation. The nonpathogenic protozoa are important to the laboratory technician only from the standpoint of clearly distinguishing them from the harmful forms.

2. The study of Chapter 2 will begin with the flagellates, to be followed by the amebae, then the sporozoans. The chapter will end with the intestinal ciliate, *Balantidium coli*. In this chapter and those to follow, the study will be limited chiefly to morphology, pathogenesis, and diagnosis.

3. Flagellates

3-1. As you study the flagellates, you will cover each genus of medical importance. They will be discussed in the order in which they appear in table 2.

3-2. **The Leishmania.** You can divide the life history of these parasites into two stages. In man and other mammals a nonflagellated stage exists which is called a leishmania-form organism (shown in fig. 1). In the sandfly vector, and when grown in culture media, the parasite lives as a flagellated leptomonas-form of organism. Figure 2 illustrates the flagellated leptomonas-form of this group of organisms. The morphology of the three presently recognized species of *Leishmania* organisms *(L. donovani, L. tropica, L. braziliensis)* is identical. They are classified on the basis of clinical manifestations and geographical distribution.

3-3. *Leishmania donovani. L. donovani* is found in many regions of Asia, Africa, Europe, South America, and Central America. Rounded, leishmania forms contain a nucleus and kinetoplast (parabasalbody plus blepharoplast) but they lack a flagellum. Leishman-Donovan bodies, as the leishmania forms are called, are widely distributed within the body of a patient with kala-azar (visceral leishmaniasis). The parasites seem to be more abundant, however, in the reticuloendothelial cells of the liver, spleen, bone marrow, and visceral lymph nodes.

3-4. The pathogenesis and symptomatology of the disease develop as follows: once the leptomonad stage is transmitted by the vector to man, the leishmaniae develop in the cells of the skin of the host. After a variable period of time, some of the organisms gain access to the blood stream or lymphatics and are transported to the viscera, where they enter fixed tissue macrophages. The parasites multiply rapidly and are found to be abundant in the spleen, liver, and bone marrow. The rapid intracellular multiplication of parasites causes an excess production of macrophages. In time this causes a decrease in neutrophils. Finally, the red bone marrow slows down the production of red cells and anemia develops. The decrease in neutrophils favors secondary bacterial infections in the unprotected host.

3-5. Laboratory diagnosis is limited to the actual demonstration of the organism. The specimen of choice is a Giemsa stained bone marrow specimen. Of course, the organisms can be demonstrated in spleen, liver, or lymph node biopsy impression smears or tissue sections of those organs. The organisms are rarely observed in peripheral blood smears. They can be cultured on NNN medium (Novy, MacNeal, Nicolle); however, this technique is of limited diagnostic value because of the time required for the organisms to grow. Impression slide smears can be stained with Giemsa or Wright stain. The culture medium and stains are also applicable to the other leishmanias to be studied.

3-6. *Leishmania tropica.* The disorder caused by this parasite is often called "Oriental Sore" or cutaneous leishmaniasis. It is common in the Middle East, and it is endemic in Mexico, Africa, India, and other parts of Asia. Sores begin as small red papules which enlarge to an inch or more in diameter. These may develop into un-

TABLE 2
OUTLINE OF PROTOZOAN PARASITES

PHYLUM PROTOZOA

SUBPHYLUM MASTIGOPHORA (Flagellates)	SUBPHYLUM SARCODINA (Amebae)	SUBPHYLUM SPOROZOA (Sporozoans)	SUBPHYLUM CILIOPHORA (Ciliates)
Leishmania donovani	*Entamoeba histolytica*	*Isospora belli*	*Balantidium coli*
Leishmania tropica	*Entamoeba hartmanni*	*Isospora hominis*	
Leishmania braziliensis	*Entamoeba coli*	*Toxoplasma gondii*	
Trypanosoma rhodesiense	*Entamoeba gingivalis*	*Plasmodium vivax*	
Trypanosoma gambiense	*Endolimax nana*	*Plasmodium falciparum*	
Trypanosoma cruzi	*Dientamoeba fragilis*	*Plasmodium malariae*	
Chilomastix mesnili	*Iodamoeba butschlii*	*Plasmodium ovale*	
Giardia lamblia			
Trichomonas vaginalis			
Trichomonas tenax			
Trichomonas hominis			

sightly ulcerating lesions if they become secondarily infected. The sores persist for a few months to a year or more.

3-7. *L. tropica* is not able to penetrate the normal unbroken skin. Infection is normally caused by the bite of the sandfly vector. This parasite can be transmitted by intimate contact or autoinoculation. Secondary bacterial infections are not uncommon. Persons who become infected with this species usually develop a life-long immunity.

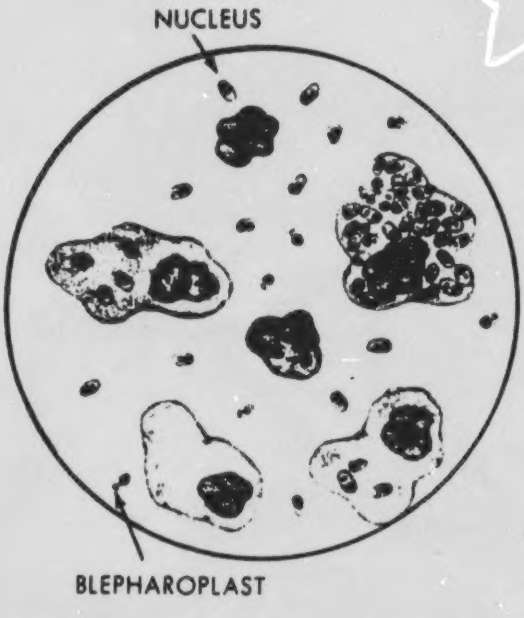


Figure 1. Leishmania: Leishmania-form organisms.

3-8. In morphology, *L. tropica* is identical to both *L. donovani* and *L. braziliensis*. Parasites of *L. tropica* are found in macrophages around the margins of the necrotic lesions. It is useless to make smears from peripheral blood in attempting to find *L. tropica*. They can be found in material from the edge of a sore. Smears prepared with this material and stained with Giemsa or Wright stain will usually show microscopic evidence of infection. If smears prove negative, then cultures on NNN or other suitable media should be completed.

3-9. *Leishmania braziliensis*. These parasites cause a disease known as South American mucocutaneous leishmaniasis, or Espundia. Initially the parasites are localized in cutaneous sores, as in the case of *L. tropica*. As the disease advances, the organisms localize in mucous membranes of the nose and pharnyx. There is a tendency for sores to spread over extensive areas and for numerous sores to appear. In later stages of the disease secondary foci occur in nasal cavities, mouth, and pharnyx; however, these generally develop several months to several years after cutaneous lesions appear. The disease occurs in large areas of tropical America from Yucatan in Mexico through Central America into northern Argentina.

3-10. Studies of this parasite suggest the possibility that there are several strains scattered through parts of South American and southern Mexico. Secondary lesions usually appear before the primary sore has healed, but they may occur

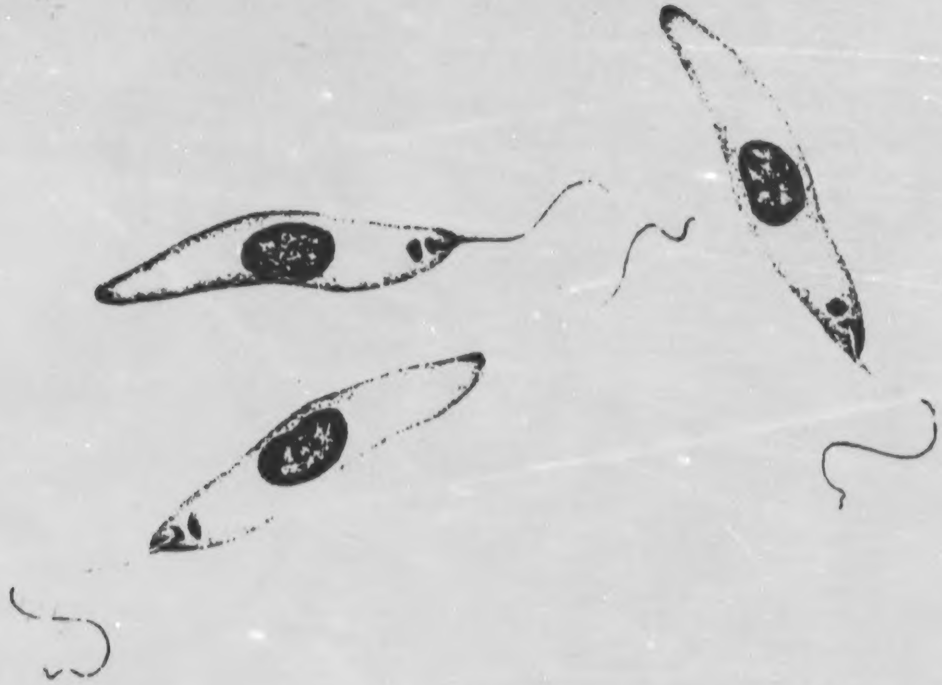


Figure 2. Leishmania: leptomonas-form organisms.

months or years afterwards. Mucocutaneous involvement results in clogging of nostrils, fetid breath, damage to the organs of smell, hearing, and sometimes sight. In advanced stages the nasal septum and surfaces of the palate are destroyed. Death frequently occurs in advanced untreated cases. This is usually due to secondary bacterial infections; however, patients may live for several years.

3-11. Laboratory procedures used for diagnosis of *L. braziliensis* include direct examination of exudates or biopsies from involved areas as mentioned for *L. donovani* and *L. tropica*. Also, cultures and serological methods may be useful. One will not find this species in peripheral blood unless the parasites are found in the local circulation close to a lesion.

3-12. **The Trypanosomes.** You will study the three trypanosomes of medical importance in the next few paragraphs. The three are *Trypanosoma rhodesiense*, *Trypanosoma gambiense*, and *Trypanosoma cruzi*. *T. rhodesiense* and *T. gambiense* produce the disease known as African sleeping sickness. As the name implies, it is restricted to the African continent. *Trypanosoma rhodesiense* and *T. gambiense* are considered to be mutants of *T. brucei*, which is a parasite of African game animals. The African trypanosomes use *Glossina* (the tsetse fly) as a vector. The organisms are morphologically indistinguishable. This supports the hypothesis that they are from the same stock. *T. cruzi* uses triatomid bugs as vectors. *T. cruzi* is restricted to the Americas. Figure 3 illustrates a typical trypanosome.

3-13. When you study the morphology of the unstained trypanosome in a wet preparation of blood or spinal fluid, the parasite appears as a colorless, slender body which measures approximately 15 to 30 microns in length. It travels with a spinning type of motion. Both the anterior flagellum and the undulating membrane can usually be observed.

3-14. The three trypanosomes are morphologically similar. A blepharoplast is located at the posterior end, from which a flagellum arises. A nucleus containing a karyosome is present, and an undulating membrane extends almost the length of the body.

3-15. *Trypanosoma rhodesiense*. *T. rhodesiense* is present in the circulating blood during febrile episodes. During nonfebrile states, the organisms are more abundant in the lymph glands. In the cases that reach the late stages of the disease, the trypanosomes may be found in the cerebrospinal fluid and within the tissues of the central nervous system. This organism is apparently more virulent than *T. gambiense*. Death may occur within 3 or 4 months following infec-

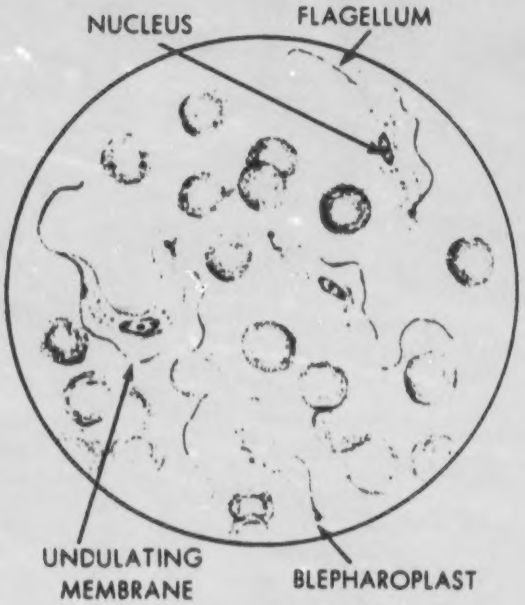


Figure 3. Typical Trypanosomes.

tion. The initial symptoms of infection are recurring fever and headache followed by weakness, enlarged glands, edema, and muscular tenderness. In the late stages there is physical depression, stupor, unconsciousness, coma, and death.

3-16. Laboratory diagnosis includes the following procedures:

- Stained blood smears.
- Wet slide preparations of blood.
- Centrifugation with wet or stained preparation of spinal fluid.
- Animal inoculation.
- Demonstration of trypanosomes in bone marrow and lymph node aspirates.

Cultivation can be attempted on Weinman's medium; however, it is difficult to get a viable culture of either *T. rhodesiense* or *T. gambiense*.

3-17. *Trypanosoma gambiense*. This species produces the disease often called Gambian sleeping sickness. It is geographically distributed in tropical West and Mid-Africa. As previously mentioned, it is transmitted through the bite of an infected tsetse fly (the biological vector). Mechanical transmission of the disease is possible if a fly bites an individual who has the disease and within a very short period of time bites a noninfected person. The transmitted parasite, in this case, does not undergo the usual multiplication in the salivary gland of the fly but is transferred directly from contaminated mouth parts. This method of transmission occurs frequently in severe epidemics.

3-18. The morphology of *T. gambiense* is indistinguishable from that of *T. rhodesiense*. Within vertebrate hosts these parasites localize first in the blood, but later they are found in greater numbers in enlarged lymph glands. In the late stages of the disease, the parasite may be found in the cerebrospinal fluid. Invasion of the central nervous system takes place later in the course of the disease than it does in infections with *T. rhodesiense*.

3-19. The Gambian disease appears milder than infections with the Rhodesian type. Initial symptoms include fever and headache recurring irregularly, followed by weakness, enlarged glands, edema, and muscular tenderness. The disease does not usually result in death. However, fatal cases do occur, particularly among patients who develop nervous system involvement and complications from other tropical diseases. The disease is diagnosed by demonstrating the parasite in blood and other body fluids using the same techniques used to diagnose *T. rhodesiense*.

3-20. *Trypanosoma cruzi*. This parasite causes an illness known as Chagas' disease. It is most prevalent in parts of Mexico and Central and South America. A few cases have been reported from Texas. Man is only one of many mammals that are susceptible to the parasite. Many domestic and wild animals are reservoirs for this organism.

3-21. *Trypanosoma cruzi* ordinarily requires a vector for transmission. Infants can acquire the parasite by prenatal infection, and the organism is occasionally transmitted by blood transfusion. The primary means of transmission is by an insect vector.

3-22. The vectors in Chagas' disease are several species of triatomid bugs commonly known as "assassin" or "kissing" bugs. As the infected bug takes a blood meal, it discharges liquid feces containing the infective trypanosome-form of the parasite, which is rubbed into the bite site, or at times actively invades mucous membranes when the feces are wiped into the eye or mouth. The infective trypanosome is engulfed by macrophages. Within macrophage cells the parasite develops into the leishmania form of the organism (similar to that in fig. 1). The parasite multiplies in the leishmania-form and produces a local swelling (chagoma). A few days later it is found in the circulating blood as a trypanosome-form organism again. The organism never multiplies in the trypanosome-form. These trypanosome-forms of the organisms enter visceral reticulo-endothelial cells, myocardial tissue, endocrine gland tissue, or almost any type of cell. Within the cell, the organism reverts to the leishmania-form. It is in this form that reproduction occurs. Eventually the cell ruptures, releasing numerous leishmania-forms of the organism which very rapidly develop into trypanosome-forms. The change is so rapid that leishmania-forms are not seen in peripheral blood. Some of the trypanosome-forms enter peripheral circulation, but most infect other tissue cells and revert to the leishmania-forms for further reproduction.

3-23. Symptomatology during the primary parasitemia (acute phase) consists of fever and a marked toxic condition. The acute phase usually persists for about 12 to 30 days. In some cases, children die during this phase. Patients who survive the acute phase develop the chronic state of the disease. Symptoms of this phase depend upon where the parasites localize. This stage may last for years. In this stage, there are usually very few trypanosomes in the circulating blood.

3-24. *T. cruzi* in the trypanosome-form is morphologically similar to the other trypanosomes, but it is different enough for you to identify the organism. You will sometimes see the trypanosome-form as a delicately spindle-shaped organism, about 20 microns long with only two or three curves in the narrow undulating membrane. There is also a short, free flagellum. Other times, the organism may appear as a considerably shorter, broader, more or less C-shaped organism, with or without a free flagellum. In its intracellular phase, *T. cruzi* is a typical leishmania-form organism, oval in shape, 1.5 to 5 microns in length, with a large nucleus and a deeply staining kinetoplast (fig. 1). In reticulo-endothelial cells, you cannot distinguish *T. cruzi* from the *Leishmanias*. A distinguishing feature to remember is that *T. cruzi* invades heart muscle cells and nerve tissue cells as a leishmania-form organism. The *Leishmanias* do not invade these tissues.

3-25. Laboratory diagnosis is made by finding the typical trypanosomes of *T. cruzi* in blood films during febrile periods. Aspirates of spleen, lymph node, liver, or bone marrow are the materials of choice for finding the leishmania-form organisms. NNN media should be inoculated at the same time the smears are made. Quite often you can culture the organisms in this manner even if there are too few to find on a stained smear. Complement fixation is sometimes a satisfactory method of diagnosis when dealing with chronic cases. In endemic areas, xeno-diagnosis is frequently employed. In this procedure a "clean" (uninfected) triatomid bug is allowed to bite the patient. If the patient is infected, the fecal material of the bug will contain the organisms after about 10 days.

3-26. In this study of the trypanosomes, note that the African trypanosomes and *T. cruzi* are found on separate continents, yet they are very closely related organisms. The next organisms that we will discuss are the intestinal parasites *Chilomastix mesnili* and *Giardia lamblia*. They are not systematically closely related; but because they form resistant cysts, it will be convenient for you to study them together.

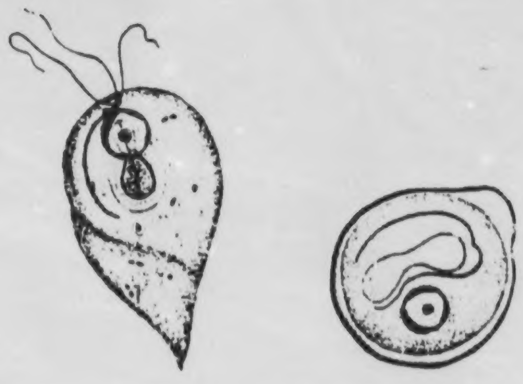

A TROPHOZOITE B CYST

Figure 4. *Chilomastix mesnilii.*

3-27. **Chilomastix and Giardia.** These organisms are flagellated, protozoan parasites of the human intestinal tract. *Chilomastix mesnili* normally inhabits the large intestine, while *Giardia lamblia* inhabits the upper small intestine. Each of these organisms exhibits actively motile trophozoites and also resistant encysted-forms. They are passed from the body in fecal material in either form, except in severe diarrhea. In severe diarrhea the predominant form found is the trophozoite. *C. mesnili* and *G. lamblia* do not produce serious intestinal disease. In fact, some authorities consider them non-pathogens. Since *G. lamblia* is one of the most frequently found human intestinal parasites, it is appropriate that you become familiar with this group.

3-28. *Chilomastix mesnili.* This parasite is found in the lumen of the large intestine and obtains food from enteric bacteria in the large intestine. It is widely distributed, but is more prevalent in warm climates.

3-29. The parasite occurs in the trophozoite and cystic stages. Freshly passed liquid stools show trophozoites that are very actively motile; they move about in a jerky, spiral fashion with directional movement. Movement is accomplished by means of flagella which can be observed in stained preparations. Figure 4A shows a drawing of the trophozoite.

3-30. The distinctive features of the trophozoite are clearly visible in hematoxylin stained preparations. Prominent internal structures include an anteriorly located nucleus and a primitive mouth (cytostome). The nucleus has a well-defined nuclear membrane. The karyosome is small, dot-like, and centrally located. Immediately above the nucleus in the anterior end of the organism there is a small group of blepharoplasts, from which three flagella extend anteriorly. A fourth extends posteriorly about one-half the length of the body. There are two curved fibrils, one on each side of the cytostome.

3-31. Stained cysts of this species are lemon shaped, as shown in figure 4B. In length they are about equal to the diameter of a red blood cell and about two-thirds as wide. The most characteristic morphological structure is a protuberance on one margin, giving the cysts the shape of a lemon. Cysts usually contain a single nucleus situated near the protuberance. Remnants of the trophozoite remaining in the cyst are the cytostome and the two fibrils.

3-32. *C. mesnili* is not pathogenic. One must be careful in laboratory examinations not to confuse the organism with *Trichomonas hominis*, which is also found in fecal specimens. Confirmation is made by examining fixed stained preparations.

3-33. *Giardia lamblia.* In fresh preparations, the trophozoite of this species is actively motile and demonstrates nondirectional movement. It resembles a longitudinally cut pear. It is about twice the diameter of a red blood cell in length, and only slightly wider than a red blood cell. It is shaped much like a saucer. The concave side forms a sucking disc by which the parasite adheres to mucous membrane surfaces.

3-34. The best way for you to study the detailed morphology of this species is to use an iron hematoxylin stained preparation. *G. lamblia* is the only bilaterally symmetrical protozoan infecting man. Figure 5A shows some of the important structures. Distinctive features that you usually find are:

a. Four pairs of flagella.

b. Two nuclei in the anterior third of the body, one on either side of the axostyles.

c. A rod-like structure lying diagonally across the center of the body that is believed to be the parabasal body.

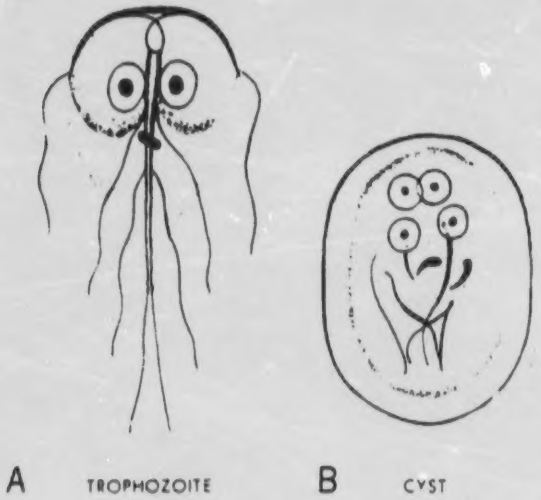


Figure 5. *Giardia lamblia.*

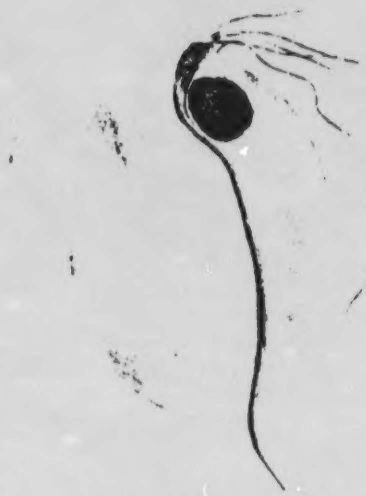


Figure 6. *Trichomonas vaginalis.*

3-35. This organism is commonly found in the cystic stage but is frequently found in the trophozoite stage in loose stools. In hematoxylin stained preparations of the cystic stage, the nuclei stand out as four "eye-like" ovid objects similar to the nuclei seen in the trophozoite. These nuclei are generally grouped near the anterior end of the organism. The remains of the axostyles also appear as curved rods running down the center of the cyst. Figure 5B shows these features in a drawing of the cyst.

3-36. *Giardia lamblia* is the most frequently diagnosed parasite in the intestine of man. It does not usually produce any illness of significance. The organism has been implicated as the cause of:

- Intestinal irritation.
- Chronic diarrhea.
- Slight epigastric pain.
- Decrease in fat absorption.
- Weight loss.

3-37. **The Trichomonads.** The trichomonads are small flagellated, pear-shaped protozoans with prominent undulating membranes. They do not form cysts and, as a result, are usually seen as actively motile trophozoites. On occasions they assume a rounded-up form that resembles a cyst.

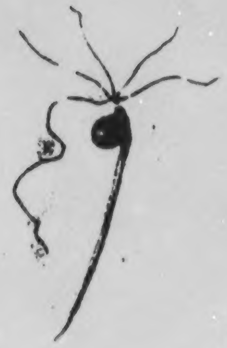


Figure 7. *Trichomonas tenax.*

They are not genuine cysts. These organisms, as a rule, thrive where there is a lack of personal hygiene. The most common human trichomonads are: *Trichomonas vaginalis*, *T. tenax*, and *T. hominis*. *T. vaginalis* is considered a mild pathogen. The other trichomonads are considered nonpathogens.

3-38. *Trichomonas vaginalis*. This species has a worldwide distribution. The organism exists only in the trophozoite stage. When found in fresh urethral or vaginal discharges, the trophozoites are readily visible under high dry magnification. The presence of flagellates in the genitourinary tract strongly suggests a diagnosis of *T. vaginalis*. No other flagellates are ordinarily found at that site in man. Figure 6 is a drawing of the organism.

3-39. The internal and external structures of *T. vaginalis* are best observed if the preparation has been stained. There are four anteriorly directed flagella arising from the blepharoplast. A fifth flagellum is directed posteriorly along the margin of the relatively short undulating membrane. It does not extend beyond the posterior limit of the membrane. The nucleus is usually elongated, with the nuclear chromation uniformly distributed. There are numerous rod-like granules evenly distributed throughout the cytoplasm. The supporting axostyle originates in the blepharoplast and terminates posteriorly in a long, free portion protruding from the body wall.

3-40. In females *T. vaginalis* causes severe irritation of the vaginal and urethral mucosa. The inflammatory condition that results can be very intense. The inflammation develops from the degeneration of the epithelium caused by large numbers of flagellates. This condition causes the increased production of vaginal secretions. In the male the flagellates may cause an irritating urethritis or prostatitis.

3-41. Laboratory diagnosis is made by the recovery and identification of the organisms from vaginal or urethral secretions. Frequently the organism is found in centrifuged urine specimens. Motile forms can be observed for about 1 hour in urine after the specimen is voided. More than 1 hour after the specimen is voided you will normally find only nonmotile organisms. To recover the parasite from genitourinary secretions collect the specimens on cotton-tipped applicator sticks. Place the applicator sticks in a small amount of normal saline. Reliable culture techniques are available and should be employed when direct smears are negative. Avoid contamination of urine by feces because *T. hominis* can be easily mistaken for *T. vaginalis*.

3-42. *Trichomonas tenax*. This trichomonad is a common parasite of the mouth. It lives in the tartar around the teeth and gingival margins. *T. tenax* is encountered in all areas of the world.

3-43. *T. tenax* has been recovered only in the trophozoite stage. It has a length of 6 to 12 microns and a granular cytoplasm. The undulating membrane is relatively short. In stained preparations, the axostyle protrudes a considerable distance beyond the posterior margin of the body. Figure 7 shows a drawing of the *T. tenax*.

3-44. *T. tenax* is of little pathological significance and is considered to be harmless. It has been incriminated, however, in a few respiratory infections and lung abscesses, probably as a secondary invader. The presence of the organism indicates very poor dental and oral hygiene.

3-45. You can recover *T. tenax* by obtaining smears from areas between the teeth, margins of the gums, and tonsils. Make plain wet mounts or stain slides of material from the mouth to isolate the parasite.

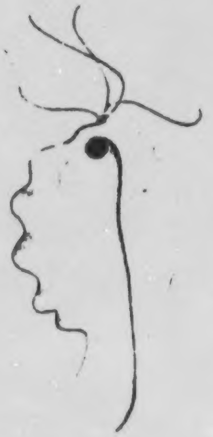


Figure 8. *Trichomonas hominis.*

3-46. *Trichomanas hominis.* As with the other trichomonads there is no known cystic stage. In fresh preparations (wet mounts) the organism exhibits a wobbly yet progressive motion which is brought about by the active vibration of the flagella and the undulating membrane. To observe the organism as shown in figure 8, the microscope light must be decreased considerably, since the parasite is very hyaline, clear, and finely granular. Specimens stained with hematoxylin show the internal and external structures very clearly.

3-47. The distinctive features of *T. hominis* are:

- Pear-shaped body.
- Three to five anteriorly directed flagella originating from the blepharoplast.
- A single, posteriorly directed flagellum which also originates from the blepharoplast and borders an undulating membrane.
- The flagellum bordering the undulating membrane extends beyond the posterior margin of the membrane.
- A prominent nucleus.

3-48. You will find motile *T. hominis* only in fresh stool preparations. This species has no known pathological significance. You must be able to identify the organism in case of contamination of urine specimens with feces. There are two features for differentiation between *T. hominis* and *T. vaginalis* when stained with hematoxylin, namely:

a. The undulating membrane of *T. hominis* is longer and the flagellum bordering that membrane extends beyond the posterior border.

b. T. hominis averages 7 to 8 microns in length, while *T. vaginalis* averages 13 microns in length.

4. Amebae

4-1. Finding and accurately identifying cysts and trophozoites of amebae in the routine examination of specimens is one of the most difficult tasks confronting the laboratory technician. One must be able to differentiate those of medical importance from those which are harmless. Our study of the amebae will be confined to the seven listed in table 2. The organisms will be discussed in the order given in table 2.

4-2. Six of these amebae inhabit the human intestinal tract, and one is found in the spaces around the teeth. Various stages that you can find in fresh specimens and in fixed preparations will be described in the section which follows.

4-3. **Entamoeba Histolytica.** This species is the most pathogenic of the amebae because of its ability to invade and destroy tissue. It has been found in populations worldwide. The organism is more prevalent in warmer climates than in cooler areas.

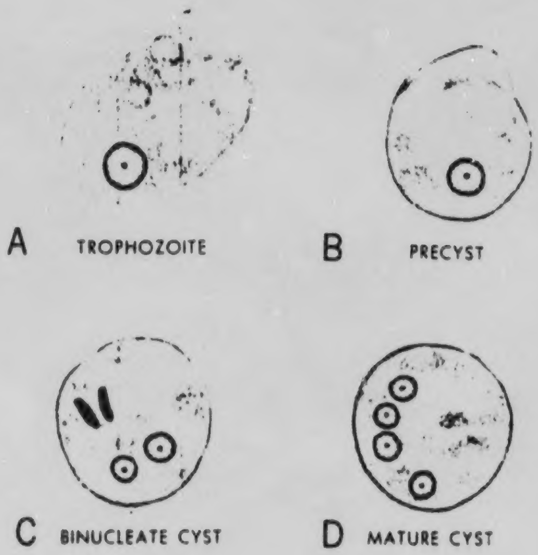


Figure 9. ***Entamoeba histolytica.***

4-4. Figure 9A represents a drawing of the trophozoite of *E histolytica.* Trophozoites are three or four times the size of a red blood cell. An actively motile trophozoite has a clear zone of ectoplasm occupying about a third of the entire cell. The remainder is composed of fine granular endoplasm in which food vacuoles and the nucleus float about. When warm, these amebae are very active. They move rapidly and extend a single, clear pseudopod in any direction. However, they do tend to travel in a straight line. Food inclusions consist of red blood cells, white blood cells, and other cellular debris. The nucleus in fresh preparations may be barely visible as a faint ring or not visible at all. In stained preparations the ectoplasm and endoplasm are not differentiated into clearly defined zones. In all stages of development, the nucleus is almost perfectly circular. The limiting membrane of the nucleus, known as the nuclear membrane, has on its inner surface a thin layer of evenly distributed chromatin granules that are bead-like in arrangement. At the center of the nucleus is a small dot-like body known as the endosome or karyosome. In the course of transition from ths active trophozoite to the dormant, cyst stage several well-defined intermediate stages can be identified.

4-5. Progressing from the trophozoite stage, the precyst shown in figure 9B is seen next. The uninucleate, binucleate, and quadrinucleate cyst (mature form) follow in succession. As the encystment process begins, pseudopods are lost and the organism rounds up. A well-defined cyst wall or protective covering is secreted as encystment advances. As the cyst develops, food inclusions are extruded and chromatin bodies of varying shapes and sizes appear. The uninucleate cyst occasionally displays a broad, oval clear area which is a glycogen vacuole. Figure 9C shows the organism as it progresses to the binucleate stage. The glycogen vacuole, when present, becomes smaller and chromatoidal material begins to congregate into fewer and more uniform masses as the cyst matures.

4-6. The mature cyst has four nuclei and is shown in figure 9D. Throughout this gradual transition the intranuclear characteristics remain unchanged. The dot-like karyosome and the peripherally arranged bead-like chromatin granules are identical in all stages of development. Glycogen vacuoles are not usually observed in the mature cyst. The principal morphological features of the mature cyst are the presence of four nuclei and rod-like chromatoidal bodies with smoothly rounded ends. In old cysts the chromatoidal bodies may be completely lacking.

4-7. If the parasite invades the intestinal tissue, the chances are very great that the organisms will go on to invade the liver, especially if the patient is not treated. Furthermore, invasion of the skin, lungs, and brain has been known to occur. Fatalities, although rare, do occur.

4-8. Identification of *E. histolytica* is not too difficult if you have the proper type of specimen. In fresh, warm stool specimens trophozoites of *E. histolytica* will be actively motile and move in a definite direction, while the trophozoites of *Entamoeba coli* will move in a sluggish manner without direction. You should not expect to find trophozoites in formed stool specimens. However, you can easily identify cysts in stained smears. You should examine liquid stools as soon as possible after elimination and make smears of any mucus that you find. Trophozoites are most often found in the mucus. Early examination is necessary because trophozoites degenerate in a very short time. The sample should be immediately preserved if it cannot be examined at once.

4-9. **Entamoeba Hartmanni.** *E. hartmanni* is the organism that has been previously known as small race *E. histolytica.* Morphologically *E. hartmanni* is exactly like *E. histolytica,* except that the cysts are less than 10μ in size, whereas, those of *E. histolytica* are greater than 10μ. It is important that you be able to make this differentiation, because *E. hartmanni* is not known to be pathogenic, whereas *E. histolytica* may produce very serious disease.

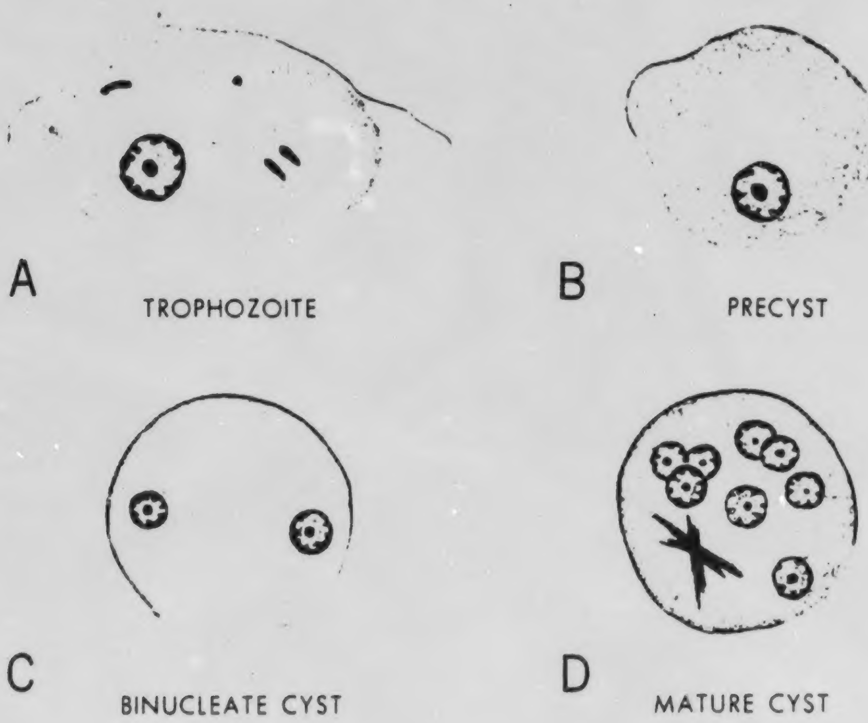


Figure 10. *Entamoeba coli.*

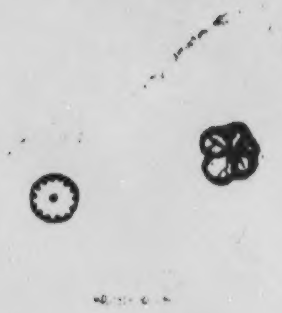


Figure 11. *Entamoeba gingivalis.*

4-10. **Entamoeba Coli.** This parasite is found in all countries of the world. There are several characteristics which differentiate this species from *E. histolytica. E. coli* is the largest of the intestinal amebae and displays numerous types of food inclusions including bacteria, starch, fragments of the host's body cells, and yeast. Movement is sluggish and nondirectional. Pseudopods are usually very narrow and composed mostly of endoplasm. They are less hyaline, much shorter, and more blunt than those of *E. histolytica.* Figure 10A depicts the trophozoite stage.

4-11. In unstained preparations the nucleus of *E. coli* is usually visible, appearing as a large refractile ring within which may be seen a small eccentric hyaline mass, the karyosome. Among the distinctive characteristics found in stained preparations is a prominent nucleus. On the inner periphery of the nuclear membrane the chromatin material is arranged in irregular masses. The karyosome is comparatively large and usually offcenter within the nucleus. As the organism progresses through the various stages in the process of encystment, several clearly recognizable transitional forms can be observed. These include precysts, binucleate cysts, quadrinucleate cysts, and mature cysts with eight nuclei.

4-12. During transition from the trophozoite to the precystic stage shown in figure 10B, food inclusions are expelled; and many small masses of chromatin material appear. A glycogen vacuole is frequently present, and it may push the nucleus off to one side of the precyst.

4-13. The next stage observed is the binucleate cyst shown in figure 10C. A rather dense mass of clycogen is often found in "unripe" cysts. It is easily demonstrated with iodine stain. As the cyst "ripens," the glycogen material appears less dense or entirely disappears.

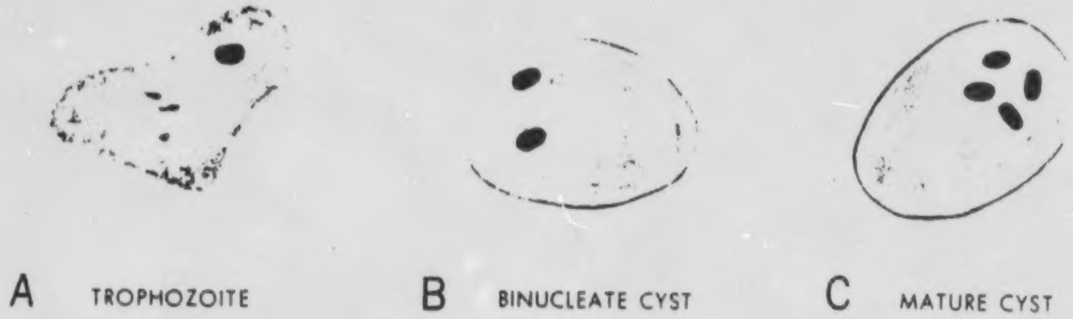
4-14. Two further nuclear divisions occur, resulting in the eight-nucleated (mature) form. This is shown in figure 10D. At this stage most of the chromatoid material has disappeared and that remaining consists of irregularly shaped "splinter-like" particles.

4-15. *E. coli* produces neither symptoms nor pathology in man. It definitely presents a problem in differentiating it from the pathogenic *E. histolytica,* especially for technicians who have not had practice in comparing the two parasites in the trophozoite stage.

4-16. **Entamoeba Gingivalis.** No cyst of *E. gingivalis* has been observed. As the parasite undergoes active movement, you can see a distinction between the ectoplasm and endoplasm. The psudopods form various shapes while the parasite is in motion. Figure 11 represents a drawing of this species in motion.

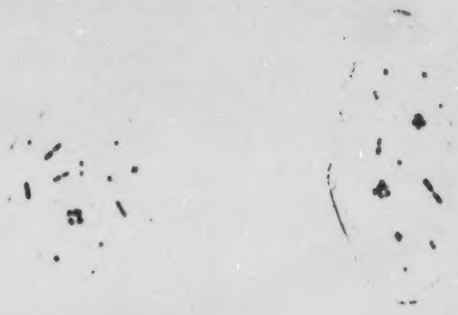
4-17. *E. gingivalis,* like *Trichomonas tenax,* is found in the tartar between the teeth. The specific identifying features are a round nucleus, a nuclear membrane lined with chromatin granules, and a small, centrally located karyosome. These features can be observed only in stained preparations.

4-18. *E. gingivalis* is not a pathogenic amebae; but its presence indicates poor oral hygiene, since the organism thrives on diseased gums and unhealthy oral conditions. You must be able to differen-



A TROPHOZOITE B BINUCLEATE CYST C MATURE CYST

Figure 12. *Endolimax nana.*



A UNINUCLEATE TROPHOZOITE

B BINUCLEATE TROPHOZOITE

Figure 13. *Dientamoeba fragilis.*

tiate *E. gingivalis* from *T. tenax* and other organisms found in the mouth.

4-19. **Endolimax Nana.** This species is small, as its name implies. It has a diameter of 6 to 15 microns. Like *E. histolytica* and *E. coli*, it is found worldwide. *E. nana* has all the stages of development that are found in *E. histolytica* and *E. coli*. Generally, movement of the trophozoite is nondirectional, and pseudopods are extruded in various directions. Figure 12A provides a drawing of the trophozoite stage.

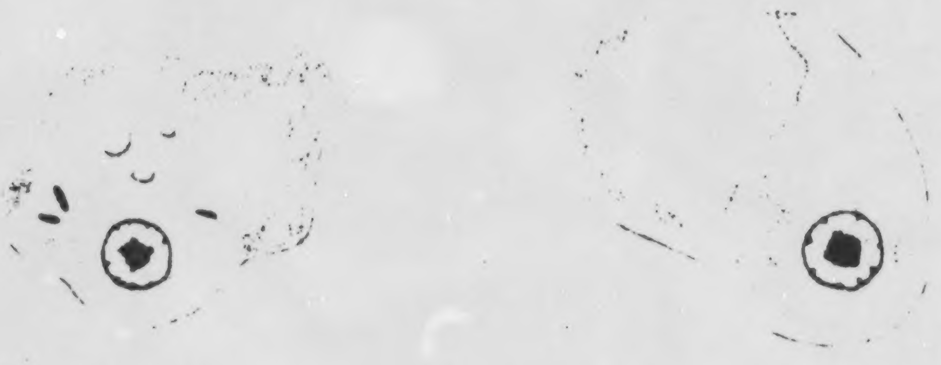
4-20. The cytoplasm of the *E. nana* trophozoites is finely granulated and has vacuoles. In stained preparations, nuclear characteristics, size, and appearance are the important points by which you can identify the species. The nuclear membrane is often invisible, and the nucleus is made conspicuous by a large eccentric karyosome. The area between the ill-defined nuclear membrane and the karyosome forms a "halo-like," narrow, clear zone free of granulation.

4-21. As the organism develops into the cyst stage, the food inclusions are lost; and the cytoplasm becomes more granular. Gradually a thin elastic cyst wall is secreted. Figure 12B demonstrates the binucleate cyst. In this stage, the nuclear characteristics are similar to those of the uninucleate form.

4-22. Figure 12C shows the appearance of the mature cyst. The mature stage is the form most frequently seen in routine fecal examinations. In stained preparations, you will find the four nuclei clustered, usually, at one pole of the cyst. The karyosomes and the surrounding nuclear membranes are oval in shape. The karyosome fills about one-half the total area of the nucleus. Cysts are sometimes round, but most often they are ovoid in shape. Chromatoidal bodies are occasionally seen in this species as small, slightly curved rods in the cytoplasm. The endoplasm often contains a number of small refractile granules.

4-23. *E. nana* is nonpathogenic, and you will find the parasite quite often during routine fecal examinations. You can see the organism in wet preparations, but it is difficult to differentiate from other species. However, the organism is easy to identify in stained preparations.

4-24. **Dientamoeba Fragilis.** Living trophozoites of this species project clear pseudopods. The movement of the organism is active and progressive. With a drop in temperature, the organism soon becomes inactive, rounds up, and quickly degenerates. The organism ruptures quickly in tap water and disintegrates. This is the only intestinal ameba of man in which no cystic stage is known. Figure 13 (A and B) shows drawings of both the uninucleate and binucleate trophozoites.



A TROPHOZOITE

B CYST

Figure 14. *Iodamoeba butschlii.*

4-25. An examination of the distinctive features of *D. fragilis* in stained preparations shows that the cytoplasm is fine granular in appearance. Food inclusions generally consist of bacteria. About 80 percent of the organisms of this species contain two nuclei. Each karyosome consists of an aggregation of four to eight separate granules. The nuclear membrane is indistinct, or invisible, and there is no chromatin lining on the inner periphery of the nuclear membrane.

4-26. In a few cases, *D. fragilis* has been suspected of producing a slight irritation of the intestinal mucosa. There is no invasion of the intestinal tissue. The incidence of this parasite in the United States is very low.

4-27. **Iodamoeba Butschlii.** *I. butschlii* is found in all countries of the world but is less common than some of the other amebae. It is the only species of intestinal ameba of man in which a single nucleus persists throughout both the trophozoite and cystic stages.

4-28. The living trophozoite, shown in figure 14A, is about one and one-half times the diameter of a red cell. Movement is sluggish; pseudopods are leaf-like and greenish yellow in appearance. They have broad clear margins. You will have difficulty in differentiating the trophozoites of *I. butschlii* from those of *E. hartmanni* if you do not pay close attention to the nuclear detail.

4-29. The cyst stage of *I. butschlii* has a single nucleus, as shown in figure 14B. The nucleus is spherical, has a rather thick membrane, and has a large karyosome which is central or somewhat eccentric. The nuclear membrane in well-stained preparations displays irregularly spaced chromatin granules which appear as a "scallop-like" fringe lining the membrane. A unique feature of this organism is the large glycogen vacuole which stains brown with iodine. The cyst is usually neither round nor oval but rather irregular with no specific form.

4-30. Laboratory identification of this species requires stained specimens. It is best for you to use both hematoxylin and iodine stained preparations. One must be careful to distinguish the parasite from *E. histolytica* and *E. hartmanni*. *I. butschlii* is usually considered to be harmless.

5. Sporozoa

5-1. The sybphylum Sporozoa includes several species that parasitize man. These are the *Toxoplasma* organisms, the cocidia and the extremely important malarial parasites.

5-2. Sporozoan organisms have an interesting and complex life cycle. They reproduce through alternating sexual and asexual cycles. The coccidia go through both the sexual and asexual cycle in one host. The malarial parasites require two hosts. At present, very little is known about the reproductive cycle of *Toxoplasma.*

5-3. In the sexual cycle, male and female gametes join to form a zygote which develops into an *oocyst.* When the oocyst reaches maturity, it ruptures and releases sporozoites. This is the sexual cycle, referred to as *sporogony.*

5-4. The asexual cycle is initiated by the sporozoites which, in developing, are transformed into trophozoites. Each trophozoite produces a *schizont.* The schizont produces a number of *merozoites.* This is the asexual cycle and is referred to as *schizogony.* Some of the merozoites will develop into male gametocytes; others will develop into female gametocytes. These unite to start the sexual cycle again.

5-5. We shall limit our discussion of the organisms in the subphylum Sporozoa to two species of *Isospora,* the *Plasmodia* (malarial parasites), and one *Toxoplasma* species *(Toxoplasma gondii).* These three groups are representative of the spo-

A IMMATURE OOCYST Isospora belli

B MATURE SPOROCYSTS Isospora hominis

Figure 15. Coccidia.

rozoa and include almost all of the organisms of the subphylum Sporozoa that parasitize man.

5-6. **The Coccidia.** *Isospora hominis* and *Isospora belli* are the only members of the subclass Coccidia that parasitize man. The organisms invade and destroy the mucosal tissue of the small intestine and cecum, causing a mucous diarrhea, low-grade fever, loss of appetite, and nausea.

5-7. Fresh fecal specimens from infected persons yield either oocysts or sporocysts, depending on which species causes the infection. If the infection is caused by *Isospora belli*, immature oocysts are passed. The immature oocyst illustrated in figure 15A looks like a thick-walled, transparent, lemon-shaped structure containing a single spherical mass of protoplasm. If you keep the stool at 25° to 30° C for several hours, the protoplasmic mass will divide into two equal masses which are the immature sporocysts. After 18–36 hours, you will find four sporozoites within each sporocyst. The sporocyst membrane is thin and very difficult to see.

5-8. In infections caused by *Isospora hominis*, you will find mature sporocysts in fresh fecal material. They are illustrated in figure 15B. You may find them singly or in pairs. The single ones that you see have been released from ripe oocysts. Some workers feel that *I. hominis* infections occur higher in the gut than *I. belli* infections and that this results in mature sporocysts being passed.

5-9. If you examine fecal material using the usual screening or concentration techniques, it is unlikely that you will find either of the organisms. This is due to the fact that in active infections, oocysts are not passed in large numbers. Also, the oocysts and sporocysts are so transparent they are usually overlooked. If a coccidia infection is suspected, then you must thoroughly examine the specimen using good concentration techniques. The concentrated sample should be stained with iodine. Oocysts and sporozoites do not take up the stain and will appear as shiny, transparent, unstained bodies surrounded by stained sediment.

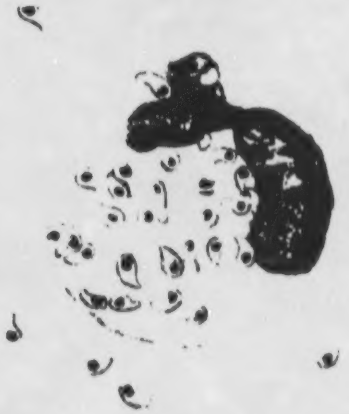


Figure 16. *Toxoplasma gondii.*

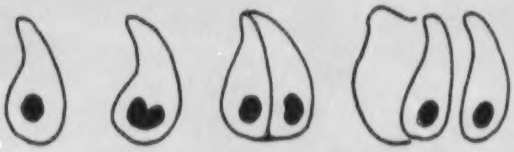


Figure 17. Reproductive Process of *T. gondii.*

5-10. **Toxoplasma Gondii.** *T. gondii* is a sporozoan parasite that commonly infects many different birds and mammals, including man. It is most prevalent in moist, warm climates, but it has been reported from all continents of the world. The organism (fig. 16) is a small comma-shaped, slightly oval body 6 to 7 microns long by 2 to 3 microns wide. When stained with Giemsa, the organism shows a delicate, light blue cytoplasm with a reddish, oval nucleus towards the broad end.

5-11. Very little is known concerning the transmission of *T. gondii*. It is known that it can be passed from an infected mother to an unborn child. It is also known that transmission occurs when improperly cooked infected flesh is eaten. Once the organism infects an animal it reproduces itself by *endodyogeny*. The process (fig. 17) begins with a longitudinal division of the nucleus. The two daughter nuclei each take on part of the cytoplasm of the parent cell. Two immature organisms develop within the parent cell; and, at a certain stage of development, the parent cell ruptures, freeing the two daughter cells.

5-12. It has been found that some strains of *T. gondii* are virulent, whereas others are avirulent. Virulent strains attack and invade fixed cells of the reticulo-endothelial system. The organisms are found free and intracellular in smears of exudates, in granulomatous tissues, and occasionally even in circulating blood. Occasional cells contain such large numbers of organisms that the cells appear cyst-like; i.e., they are pseudocysts. In humans, avirulent strains are usually discovered as incidental findings in autopsy specimens. Avirulent strains are found in thick-walled, true cysts. The cysts, tightly packed with organisms, are most frequently found in the brain and spinal cord. The next most common site is skeletal muscle.

5-13. On Giemsa stained smears you may have difficulty differentiating the various stages of *Toxoplasma* from some similar organisms. Individual *Toxoplasma* organisms are very similar to leishmania-form organisms. However, the leishmania-form organisms measure only 2 to 3 microns in length while the *Toxoplasma* organisms measure 6 to 7 microns in length. Also, the leishmania-form organisms have a distinct parabasal body that is not present in *Toxoplasma* organisms. Both are primarily intracellular organisms; but a few of them may, at times, be found free. Cells packed with leishmania-form organisms closely resemble the pseudocysts of *Toxoplasma*. The fungus *Histoplasma capsulatum* is another primarily intracellular organism that is easily confused with *Toxoplasma*. *H. capsulatum* appears in the tissues as small, oval, encapsulated yeast-like cells, 3 to 5 microns in diameter. The organisms have a central stained mass that is surrounded by a clear zone, but there is no true nucleus and there is no para-

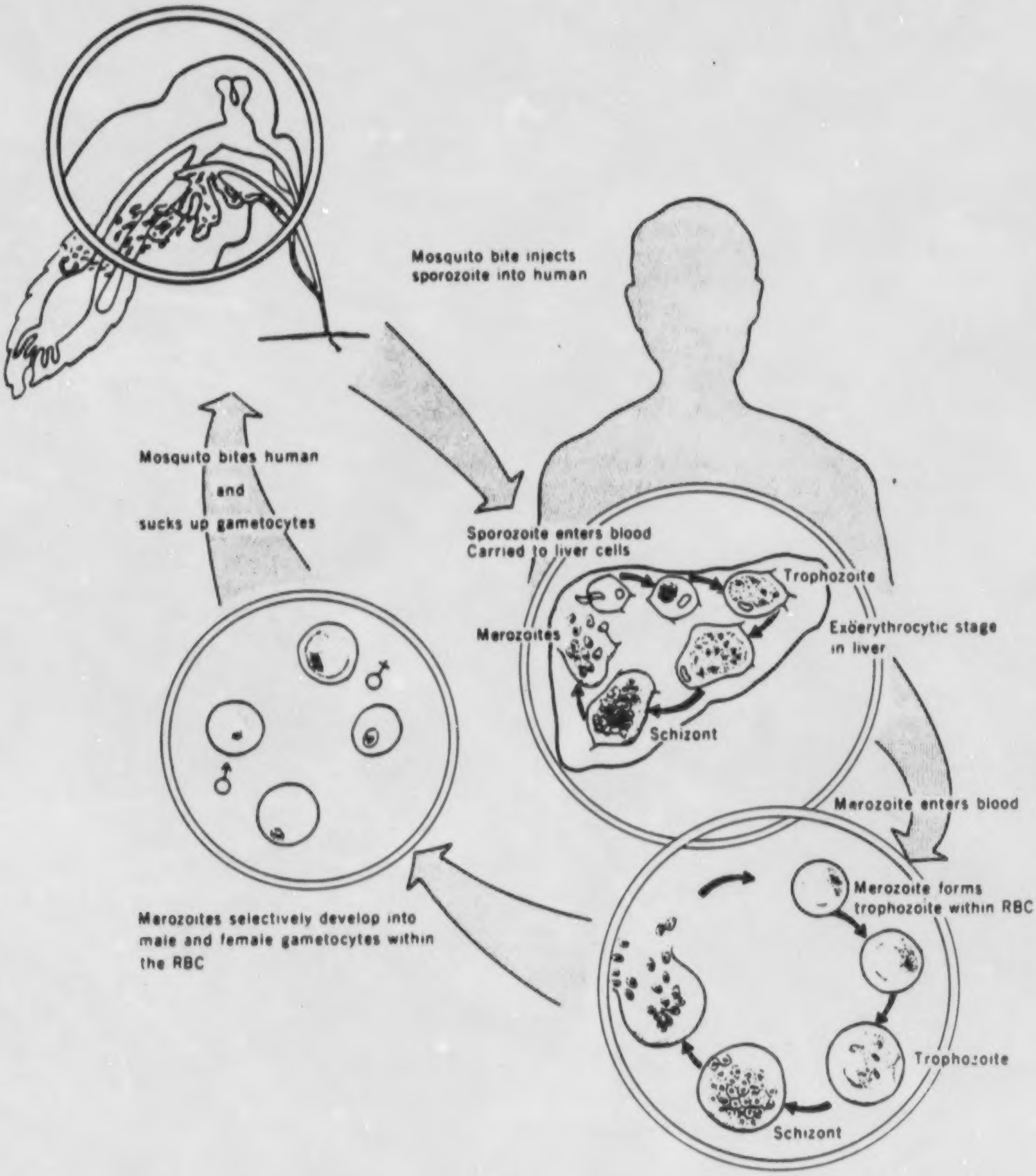

Mosquito bite injects sporozoite into human
Mosquito bites human
and
sucks up gametocytes
Sporozoite enters blood
Carried to liver cells
Trophozoite
Merozoites
Exoerythrocytic stage in liver
Schizont
♂
♀
Merozoite enters blood
Merozoite forms trophozoite within RBC
Merozoites selectively develop into male and female gametocytes within the RBC
Trophozoite
Schizont

Figure 18. Asexual cycle of plasmodia.

basal body present. *H. capsulatum* stains red with PAS while leishmania-form organisms and *Toxoplasma* organisms are completely PAS negative. The thick-walled, true cysts or *Toxoplasma* are strongly PAS positive; i.e., stain magenta.

5-14. In cases in which the parasite does not destroy tissue or produce some detectable effect, a serological test may be of value as an aid in diagnosis. The most often requested immunologic procedure is the Sabin-Feldman Dye test. This test follows the procedure briefly described below:

a. Dilute serum four-fold.

b. Harvest stock culture *Toxoplasma* organisms from 3-day infected mice. Organisms must be collected prior to antibody formation by the mice.

c. Add a standardized number of organisms to each tube of diluted serum.

d. Add dye.

e. Positive test: organisms are not stained by the dye. Anti-Toxoplasma antibodies are present and staining is inhibited.

f. Negative test: organisms are stained. Anti-Toxoplasma antibodies are not present and staining is not inhibited.

This immunologic test requires live organisms and is performed at the Communicable Disease Center of the U. S. Public Health Service.

5-15. **The Plasmodia.** Malaria, a very debilitating disease, has plagued man throughout recorded history. It was prevalent during the Greek and Roman empires. The disease was responsible for over 25 percent of all hospital admissions during the Civil War and the Spanish-American War. Epidemics of malaria severely hampered Allied operations in the Pacific and Asian theaters of World War II. American troops stationed in Korea during the police action of the early 1950's were exposed to its ravages. Recently, it has created significant problems among U.S. Forces stationed in Southeast Asia.

5-16. Many different kinds of birds and mammals can contract malaria. However, specific species of plasmodia infect specific animals. The species are similar in many respects, but each possesses qualities or characteristics that differentiate it from the others. Before you study the individual characteristics of each of these species, you should familiarize yourself with the way these organisms reproduce and how they are transferred to man.

5-17. The reproductive cycle of the malarial parasite is similar to that of other members of the subphylum Sporozoa. It consists of alternating sexual and asexual cycles. In contrast to the sexual and asexual cycles of *Isospora* that take place in a *single* host, the sexual and asexual cycles of the plasmodia take place in two separate hosts: man and mosquitoes. The asexual portion of the cycle that takes place in man is schematically illustrated in figure 18.

5-18. *Intrahuman asexual cycle of reproduction.* The disease is started in humans as a result of being bitten by an infected female *Anopheles* mosquito. The mosquito needs a blood meal to nourish herself prior to laying eggs. After the mosquito inserts its proboscis into a capillary, it pumps saliva into the capillary to prevent coagulation of the blood. Sporozoites from the salivary glands are deposited into the person's circulation.

5-19. Once sporozoites get into the circulation, they make their way to the liver, where they invade parenchyma cells. It has been estimated that within 30 minutes after a bite the sporozoites are completely removed from the blood.

5-20. In the liver the parasite begins the *exoerythrocytic stage.* Here the sporozoites mature into *schizonts.* The schizonts develop daughter merozoites. Some of the merozoites re-enter other parenchymal cells in the liver and start the exoerytherocytic phase again; others enter the circulation and invade red blood cells.

5-21. The series of events that go on within red blood cells is referred to as the *erythrocytic stage.* The merozoite, after entering the red blood cell, develops into an ameba-like trophozoite. The trophozoite ingests the hemoglobin of the cell. The protein portion of hemoglobin is used by the parasite as nourishment; the iron portion is not used. This iron collects in the parasite, and you see it as dark granules which are referred to as *malarial pigment.*

5-22. The trophozoite continues to develop until a schizont is produced. This schizont develops numerous merozoites which are released into the circulation. Some of these merozoites enter other red blood cells and start the erythrocytic stage again. The other merozoites enter red blood cells and develop into gametocytes. The parasites are sucked up in the blood meal of a mosquito. Within the mosquito, the parasite reproduces through the sexual cycle as described below and illustrated in figure 19.

5-23. *Intramosquito sexual cycle of reproduction.* Gametocytes sucked up in the blood meal are differentiated into male microgametocytes and female macrogametocytes. They are transported to the mosquito's mid-gut. Each microgametocyte eventually produces several flagellated microgametes (male sex cells). Each macrogametocyte is transformed into a single macrogamete (oocyte), which is an unfertilized female sex cell. The union of microgamete and oocyte results in a free-swimming *ookinete,* which migrates into the stomach wall and becomes an encysted oocyst. The oocyst produces within itself numerous sporozoites. When ripe, the oocyst ruptures and releases the sporozoites into the body cavity of the mosquito. The sporozoites migrate to the salivary glands, from which they escape when the mosquito feeds.

5-24. Study of the reproductive cycle of malarial parasites in man and the mosquito has contributed greatly to efforts to control the disease. The peculiar rhythmic fevers seen in malaria coincide with the rupture of red blood cells and the release of merozoites from mature schizonts. The period between fever peaks varies according to which species causes the infection. Table 3 gives the time intervals of the fever spikes for each of the species, along with a comparison of several other features that will aid you in differentiating them.

5-25. In table 3 you will notice that the four species of human malaria parasites are:

- *Plasmodium vivax*
- *Plasmodium falciparum.*
- *Plasmodium malariae.*
- *Plasmodium ovale.*

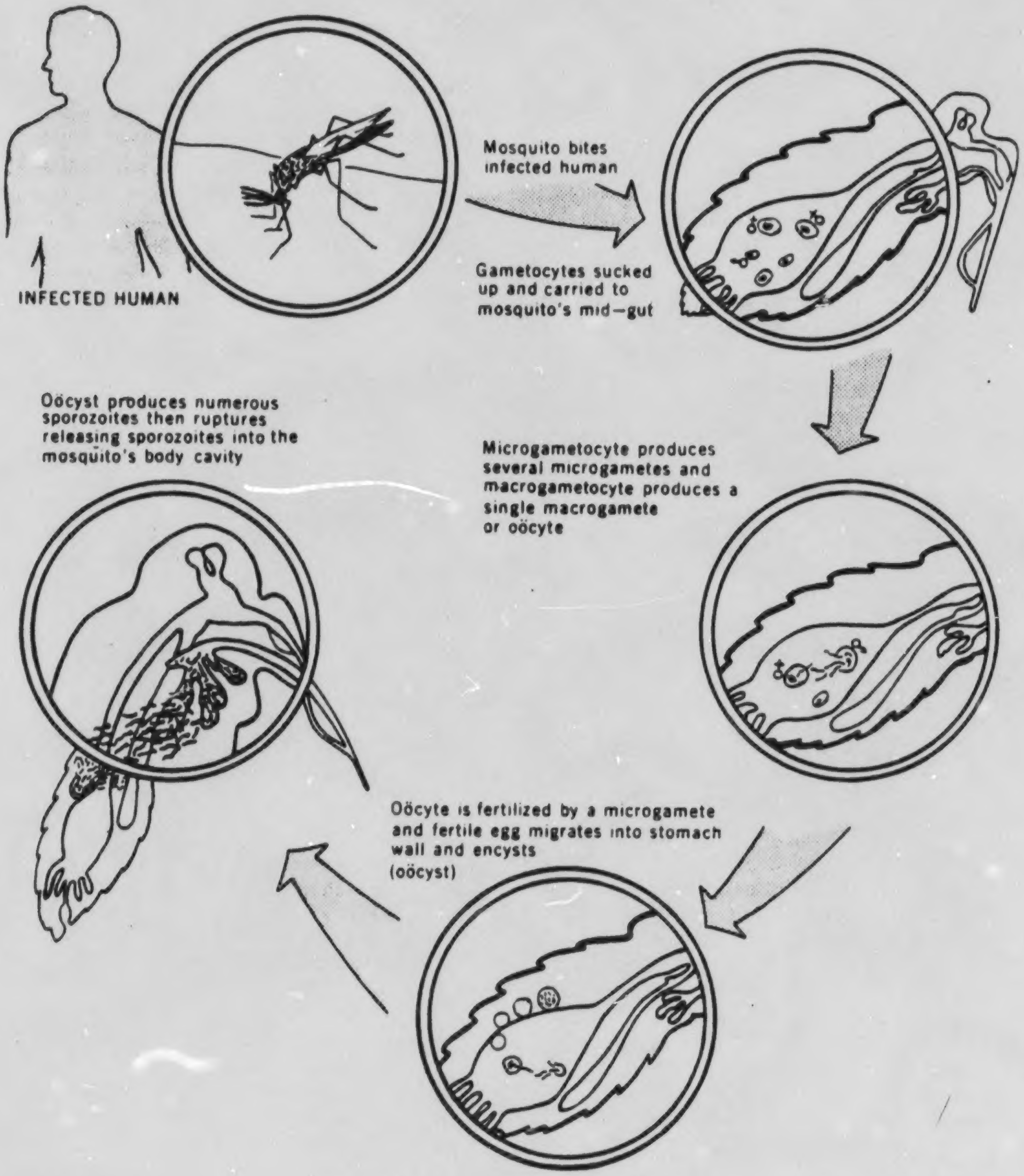

INFECTED HUMAN
Mosquito bites infected human
Gametocytes sucked up and carried to mosquito's mid-gut
Microgametocyte produces several microgametes and macrogametocyte produces a single macrogamete or oöcyte
Oöcyte is fertilized by a microgamete and fertile egg migrates into stomach wall and encysts (oöcyst)
Oöcyst produces numerous sporozoites then ruptures releasing sporozoites into the mosquito's body cavity

Figure 19. Sexual cycle of plasmodia.

TABLE 3
CHARACTERISTICS OF PLASMODIA SPECIES

	Duration of Asexual Cycle	Stages Seen in Peripheral Blood	Shüffner Granules	Appearance of Trophozoite	Malarial Pigment	Number of Merozoites in Schizont
P. vivax	48 hours	All	Yes	Very Ameboid	Light and Diffuse	Usually 12-18 (Could be 12-24)
P. malariae	72 hours	All	No	Compact, Slightly Ameboid	Very Dense	Usually 8 (Could be 6-12)
P. ovale	48 hours	All	Yes	Slightly Ameboid	Light and Diffuse	Usually 8 (Could be 6-12)
P. falciparum	48 hours	Rings, Large Gametocytes	No	Rare in Peripheral Blood	Dense in Gametocyte	Rare in Peripheral Blood

The morphological features of each is covered in the following discussion.

5-26. *Plasmodium vivax*. This species is the most widely distributed of the *Plasmodium* parasites and is found in all tropical zones of the world. The disease caused by an infection of *P. vivax* is called "tertian" malaria, a term that refers to the 48-hour period required for schizonic development in the blood to take place. Fever spikes occur at 48-hour intervals (or every third day).

5-27. Morphologically, the stages of its development may be summarized as follows:

a. The young trophozoite (ring form) usually has a large chromatin dot. The cytoplasmic circle is large and pale blue in color.

b. A growing trophozoite has an irregular outline with several finger-like pseudopodia. Fine, yellow-brown granules (malaria pigment) may be present in the cytoplasm, and the chromatin dots are single and compact.

c. In the large trophozoite, the parasite practically fills the enlarged red blood cell. Chromatin is seen as an abundant loose or compact mass.

d. Chromatin in an immature schizont is divided into two or more irregular masses. Pigment is generally clumped into several large particles.

e. A mature schizont or segmenter is divided into 12 to 24 merozoites with oval nuclei surrounded by a similarly shaped mass of cytoplasm.

f. The macrogametocyte is circular or ovoid, with homogenous cytoplasm that has no vacuoles. The chromatin dot is single, small, compact, dark red in color, and eccentric in position.

g. Usually the microgametocyte is circular when fully mature. Cytoplasm stains light blue and the chromatin mass is large, diffuse, light red in color, and located at or near the center of the organism.

5-28. Specific features of *P. vivax* can be observed in the drawings in foldout 1 (FOs 1 thru 3 located in back of volume). The drawings show the appearance of the various stages as seen in the red blood cells on stained preparations.

5-29. *Plasmodium falciparum*. Parasites of this species produce a disease called "malignant tertian" or estivo-autumnal (Summer-Autumn) malaria. The "ring forms" are smaller than those of the other species described in this chapter. Foldout 2 illustrates the appearance of *P. falciparum* in peripheral blood. *P. falciparum* is found in many tropical countries.

5-30. Some features of *P. falciparum* are:

a. Ring forms with single or double chromatin dots are often seen in peripheral smears. Multiple rings may be seen. This form and the gametocyte are the only stages normally found in the peripheral blood.

b. Trophozoites are rarely observed in peripheral blood. The cytoplasm appears compact and light blue in color. Very dark pigment is scattered throughout the cytoplasm.

c. Presegmenting schizonts are rarely observed in peripheral blood. If found, the parasite is small; and its pigment is usually clumped in one small, dark mass.

d. A mature schizont, or segmenter, is rarely seen in peripheral blood. It is divided into 8 to 32 merozoites and fills about two-thirds of a normal-sized blood cell.

e. The macrogametocyte is a long, slender, sausage-shape, with a concentrated mass of dark pigment near the center surrounding a dark red chromatin mass.

f. The cytoplasm of the microgametocyte is generally paler in color than in the macrogametocyte. The parasite is broader, shorter, and has more rounded ends than the macrogametocyte. Heavy granules of pigment are present.

5-31. *Plasmodium malariae*. Organisms of this species require about 72 hours to complete the cycle in man. The term "quartan malaria" is often used to denote an infection with this species of organism. It is not as prevalent, nor is it as widely distributed, as *P. vivax* and *P. falciparum*. The parasite is illustrated in foldout 3.

5-32. *P. malariae* presents the following appearance in stained smears:

a. The ring form contains a single, heavy chromatin dot. The cytoplasmic circle is small and compact.

b. Cytoplasm in the growing trophozoite is compact and frequently in the form of a trapezoidal-shaped band across the center of the red cell. Coarse, dark brown, or black pigment granules are often present.

c. The large trophozoite fills, or almost fills, a normal-sized red blood cell. Pigment granules are large, dark, and generally peripherally arranged. The cytoplasm is dense, dark blue, and often in the form of a broad band.

d. The presegmenting schizont has the chromatin divided into a number of masses. The cytoplasm is dense, dark blue in color, and the pigment is dark and evenly distributed throughout the parasite.

e. A mature schizont, or segmenter, has 6 to 12 merozoites, usually in a single circle surrounding a large clump of hemozoin (decomposed hemoglobin) granules. This is called the "rosette" or "daisy" form.

f. The macrogametocyte is circular or ovoid and regular. The cytoplasm is dense, dark blue, and contains abundant, coarse, dark pigment. It has chromatin which is similar to that in *P. vivax*.

5-33. *Plasmodium ovale*. Incidence of this parasite is very low. The parasite is almost completely limited to parts of West Africa. All of the stages found in *P. vivax* and *P. malariae* are also found in *P. ovale*.

5-34. In certain respects, an infection of *P. ovale* resembles *P. vivax;* in other respects it is more like *P. malariae*. The erythrocytic cycle requires 48 hours. Infected cells contain Schuffner's stippling to a more marked degree in those infected with *P. vivax*.

5-35. As the trophozoite matures, the cytoplasm continues to be relatively condensed like *P. malariae*. The red blood cells that are infected become oval shaped. They may be of normal size, or they may be slightly enlarged, but not usually paler than normal. There is not much pigment, and when present it is light brown. The mature schizont contains 6 to 12 merozoites which surround a central mass of pigment. The gametocytes are very much like those of *P. malariae;* but they can be differentiated in thin blood films by the presence of Schuffner's granules in the cells. Differentiation from the same stage of *P. vivax* is made on the basis of the denser and more compact makeup of *P. ovale*.

5-36. In studying the history and pathology of malaria, you will find that the disease has been a problem to mankind throughout the centuries. The disease can produce allergic manifestations; anemia; congestion of capillaries; enlargement of the spleen; and congestion of the kidneys, stomach, and intestines. It also produces enlargement, congestion, and pigmentation of the liver; a decrease in the functioning of the bone marrow; and fatty degeneration of the heart. It has been estimated that almost 100 million persons are afflicted with malaria. Of the species of malaria that infect man, *P. falciparum* causes the most severe disease.

5-37. Accurate diagnosis of malaria and identification of the species can be made only by laboratory studies. There are certain steps the technician might follow to improve his success in finding the organisms. These are:

(1) Prepare both thick and thin blood smear preparations.

(2) Use slides that are chemically clean.

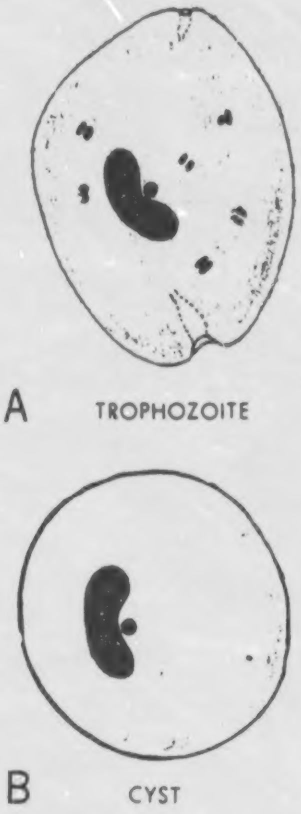


Figure 20. *Balantidium coli.*

(3) Include Giemsa stain in addition to Wright or other stains.

(4) Keep prepared slides protected from damage by insects.

(5) Collect blood smears from the patient during and immediately following episodes of fever.

(6) If malaria organisms are not found initially, continue the collection of smears for several days.

6. Ciliates

6-1. Species of the ciliated protozoa are conspicuous because of the hair-like cilia which covers their bodies. They are found in both vertebrate and invertebrate animals. Wave-like movements of the cilia provide the organisms a means of locomotion. Only one species, *Balantidium coli*, is of medical significance.

6-2. *B. coli* is the largest protozoan of man, and it exists in both the trophozoite and cyst stage. The species inhabits the large intestine of man and other animals. The motile trophozoite, found in fresh preparations, is large, oval, and slightly greenish in color. They display directional, rapid, and smooth gliding movements. On one side of the anterior end is a deep cleft which is the cytostome or primitive mouth.

6-3. Figure 20,A is a drawing of the trophozoite stage. The outer surface of the body is covered with a delicate pellicle, on the surface of which are implanted numerous short cilia in regular rows. Food vacuoles are very numerous and consist almost entirely of starch. In living trophozoites one small anterior and one large posterior excretory vacuole can be seen.

6-4. In stained specimens of trophozoites, the longitudinal rows of cilia stand out prominently. The most prominent internal structures are the two nuclei. The large kidney-shaped nucleus is called the macronucleus. There is a small dot-like micronucleus which lies nestled within the concave portion of the macronucleus. This species is a heavy feeder, and the cytoplasm is usually filled with food inclusions.

6-5. The cyst stage of *B. coli* is not often found. When you see them on a stained smear, they will be large, double-walled spherical bodies about 55 microns in diameter. The cytoplasm of the cyst is dense but uniformly granulated and lacks food inclusions. The macronucleus and micronucleus stain deeply, and they are identical to those seen in the trophozoite. An example of the cyst stage is shown in figure 20,B.

6-6. Medical experts disagree on the extent of pathology caused by *B. coli*. All agree that it is a pathogen. In a majority of cases, it causes diarrhea. In a few cases, it has been known to invade extra-intestinal sites, producing severe disease and death. Laboratory identification should cause you no special problem, because of the large size and shape of the organism.

334

Platyhelminthes Infecting Man

THIS CHAPTER will be concerned with selected members of the Phylum Platyhelminthes (flatworms). The flatworms are among the oldest parasites known to man. The Class Trematoda (flukes) and the Class Cestoidea (tapeworms) contain the flatworms of medical importance. The parasites of these two classes are listed in table 4. The worms do not have a circulatory system or a body cavity, but they do have a nervous system and a highly refined method of reproduction. The flatworms vary greatly in shape; most flukes resemble a broad leaf, while the tapeworms resemble a ribbon. A fluke develops as a single unsegmented unit, whereas a tapeworm develops as chain-like segments called *proglottids*.

2. First to be considered are the trematodes (flukes). Varous species of flukes establish themselves in specific organs, including the lungs, liver, intestine, and certain blood vessels in the mesenteries and bladder. Adult flukes, other than those which inhabit the intestine, are rarely eliminated from the body because of their location. Depending on the species of fluke, eggs may be recovered from sputum, feces, or urine.

3. The cestodes (tapeworms) will be discussed in the second half of this chapter. These worms belong to the Class Cestoidea. All members of the Class Cestoidea are parasitic. The adults are intestinal parasites of vertebrates, and their eggs are passed from the host in feces.

4. While studying this chapter, keep in mind that flotation techniques are not recommended for the detection of the eggs of these parasites. The specific gravity of flatworm eggs is too high for them to be floated consistently with flotation techniques that you use for other types of eggs. The eggs can be easily recovered if you employ direct fecal films, sedimentation techniques, or acid-ether concentration techniques. Unless it is stated otherwise in the discussion of the individual parasite, you should use the techniques mentioned above.

7. Trematodes (Flukes)

7-1. In order for you to understand flukes, you must acquaint yourself with their anatomy and morphology. Figures 21 and 22 illustrate the important anatomical features of both immature and mature flukes. To follow the life cycle of these parasites, you will have to be familiar with the terminology used. Cercariae, hermaphroditic, metacercariae, miracidium, operculum, rediae, schistosome, and sporocyst are some of the descriptive terms you should know.

7-2. **Schistosomes (Blood Flukes).** Trematodes that live in the blood stream of man are called schistosomes, or blood flukes. These worms live in pairs in the venules of specific areas. Schistosomes are unique among the flukes because the males and females are separate worms. The other flukes of man are hemaphroditic, and each individual worm has both male and female sex organs. The schistosomes are the cause of a chronic and progressive disease. They may live for 30 years or longer in a human host.

7-3. *Schistosoma mansoni.* This organism, also known as Manson's blood fluke, causes the disease variously known as Manson's schistosomiasis, intestinal schistosomiasis, or schistosomiasis mansoni. It is found chiefly in eastern and central Africa, the Middle East, the Caribbean area, and South America as far south as Brazil. Man is the definitive host, and snails of several genera are intermediate hosts.

7-4. The life cycle begins with the interlocked male and female *S. mansoni* (fig. 23) in a small venule of the mesentery of the large intestine. The male worm has a ventrally located sex canal called the gynecophoral canal. The lateral margins of the male fold around the female and hold her in the canal. After insemination, the female migrates to the end of the venule to lay eggs. Through lytic action, and by means of a sharp point on the shell, eggs are able to penetrate the small blood vessels and tissues of the mesentery, eventually reaching

353

TABLE 4
PHYLUM PLATYHELMINTHES

CLASS TREMATODA (Flukes)	CLASS CESTOIDEA (Tapeworms)
Schistosoma mansoni	*Diphyllobothrium latum*
Schistosoma japonicum	*Taenia solium*
Schistosoma haematobium	*Taenia saginata*
Fasciola hepatica	*Hymenolepis nana*
Dicrocoelium dendriticum	*Hymenolepis diminuta*
Clonorchis senensis	*Dipylidium caninum*
Opisthorchis felineus	
Opisthorchis viverrini	
Fasciolopsis buski	
Heterophyes heterophyes	
Metagonimus yokogawai	
Paragonimus westermani	

the lumen of the intestine, where they are expelled with feces.

7-5. The eggs contain a ciliated *miracidium* which escapes from the shell when the eggs come in contact with water. The miracidium seeks out and penetrates an appropriate snail. In the snail, two asexual generations of sporocysts are produced, the second generation sporocyst produces cercariae. The cercariae escape from the snail and swim about in the water. On contact with man, they penetrate the skin and migrate through the bloodstream to the mesenteric venules. Cercariae require an incubation period of 7 to 8 weeks to develop into adult male and female worms. After the females mature and are inseminated, they pass eggs, thus completing the cycle.

7-6. Adult *S. mansoni* and *Schistosoma haematobium* are morphologically similar. The male fluke is about 12 mm. long and possesses six to nine testes. The body of the male is flattened and folded ventrally to form the sex canal in which the female is held. The female is longer than the male (16 mm. as compared with 12 mm.), threadlike, and has an anteriorally located ovary. The outer surface of the adult *S. mansoni* is covered with minute sensory papillae, known as tuberosities. Figure 24 illustrates a mature egg. The egg ranges in size from 114 to 175 microns long by 45 to 68 microns wide. It is narrow and rounded at the anterior end with a more broadly rounded posterior end. The most noticeable morphological feature is the long, sharp, lateral spine on the posterior third of the egg. The eggs have a non-operculate shell. When mature, they are yellow brown in color, transparent, and contain a ciliated miracidium that is fully developed.

7-7. *S. mansoni* causes dysenteric symptoms with bloody mucus, and in old chronic infections, polypoid growths develop within the bowel. The latter condition is due to the proliferation of tissue about masses of disintegrated eggs which failed to break through into the intestinal lumen. The rectum and large intestine are the organs and tissues most seriously affected by the infection. Eggs are frequently carried through the portal blood vessels to the liver, where they filter out into the periportal tissue and cause cirrhosis of the liver.

7-8. Laboratory diagnosis of *S. mansoni* depends upon finding the characteristic eggs in fecal specimens. The number of eggs you find in a direct fecal smear parallels, to some degree, the severity of the infection. Appropriate concentration methods such as sedimentation, or acid-ether-formalin concentration are recommended. Complement-fixation, slide flocculation, and fluorescent antibody tests are the methods you should use when you do not find eggs in fecal specimens from suspected cases.

7-9. *Schistosoma japonicum.* This parasite is frequently referred to as the Oriental blood fluke. *S. japonicum* is restricted to the Far East: Japan, China, Formosa, and the Philippines. Many mammals—including dogs, cats, horses, cattle, rats, and mice—act as reservoir hosts. Snails of the Genus *Oncomelania* are the intermediate hosts.

7-10. The life cycle of *S. japonicum* is essentially the same as that of *S. mansoni.* The adult worms of *S. japonicum* inhabit the smaller venules of the messentery of the small intestine, whereas those of *S. mansoni* inhabit the venules of the mesentery of the large intestine. Therefore, the small intestine is more seriously affected in cases of *S.*

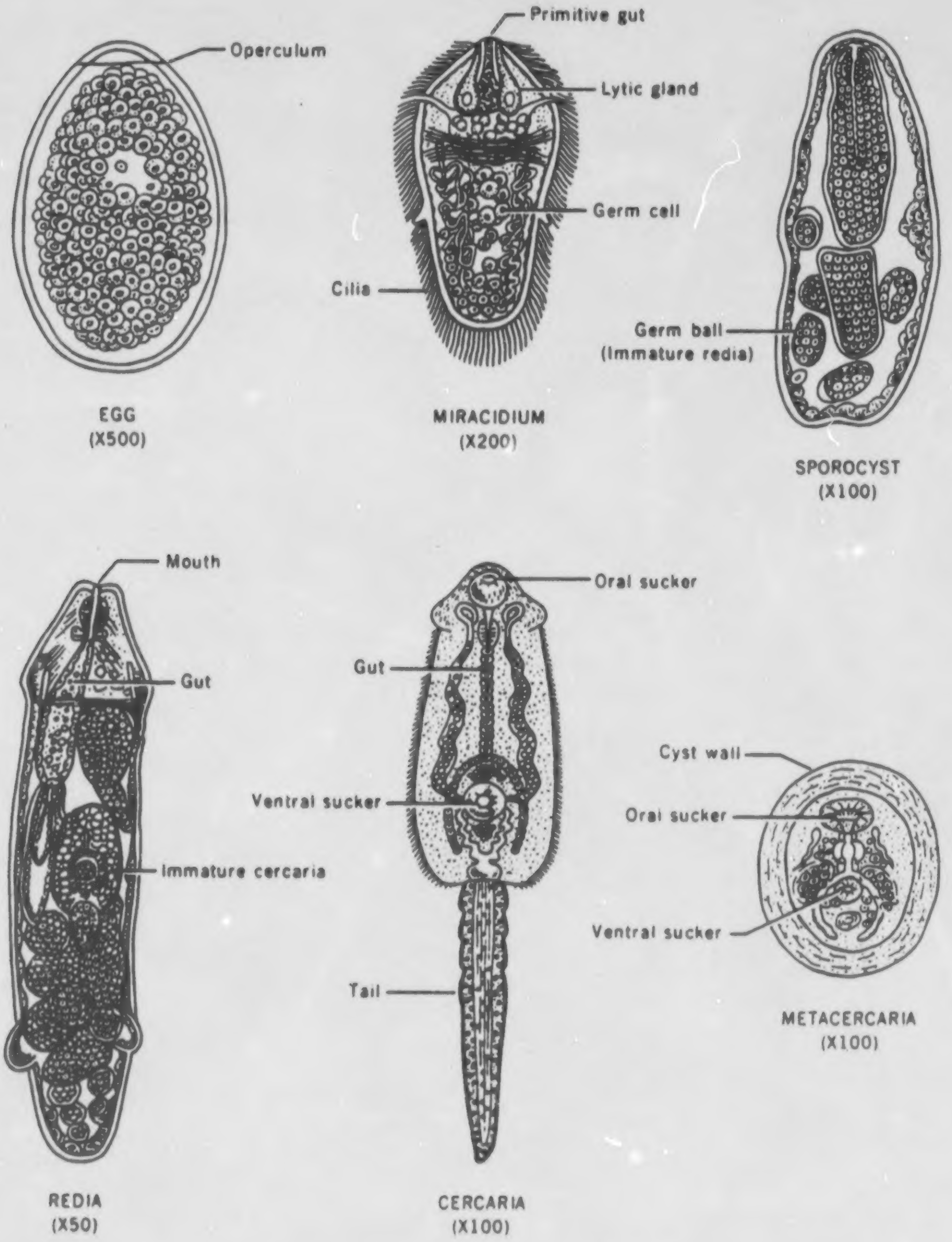

Operculum
EGG
(X500)
Primitive gut
Lytic gland
Germ cell
Cilia
MIRACIDIUM
(X200)
Germ ball
(Immature redia)
SPOROCYST
(X100)
Mouth
Gut
Immature cercaria
REDIA
(X50)
Oral sucker
Gut
Ventral sucker
Tail
CERCARIA
(X100)
Cyst wall
Oral sucker
Ventral sucker
METACERCARIA
(X100)

Figure 21. Immature stages of flukes.

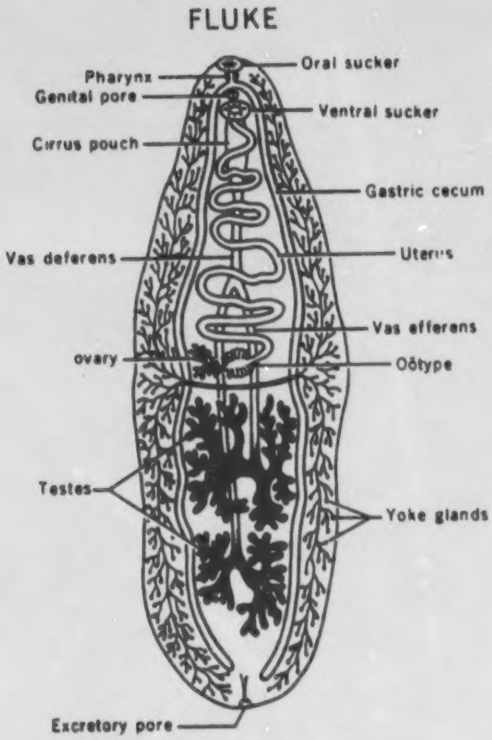

FLUKE
Pharynx
Genital pore
Oral sucker
Ventral sucker
Cirrus pouch
Gastric cecum
Vas deferens
Uterus
Vas efferens
ovary
Oötype
Testes
Yoke glands
Excretory pore

Figure 22. **Mature fluke.**

japonicum, whereas the large intestine is more seriously affected in cases of *S. mansoni*.

7-11. The adult male of *S. japonicum* is 12 to 20 mm. long. The integument is not tuberculated, but it is covered with minute spines, especially in the locality of the suckers and the gynecophoral canal. There are seven testes in the male *S. japonicum*. The female is about 26 mm. long, and the integument is also covered with minute spines. The ovary is just below the center of the body, and the vitelline (yolk-producing) glands are limited to the lateral margins of the distal quarter of the body. The uterus consists of a long, straight tube containing up to 50 eggs.

7-12. The eggs (fig. 25) are smaller than the eggs of the other species of schistosomes. They measure about 70 to 100 microns by 50 to 65 microns. You can see a wart-like thickening near the posterior end when the egg is positioned properly.

7-13. The development of the pathological effects caused by *S. japonicum* may be divided into three distinct stages:

(1) Period of incubation.
(2) Period of egg laying and extrusion.
(3) Period of proliferation and tissue repair.

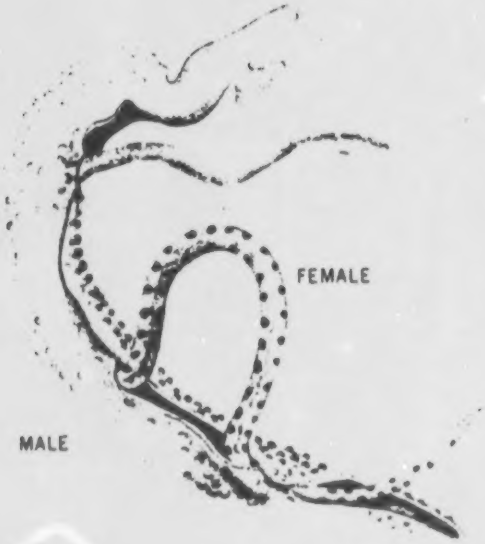

FEMALE
MALE

Figure 23. **Male and female *Schistosoma mansoni*.**

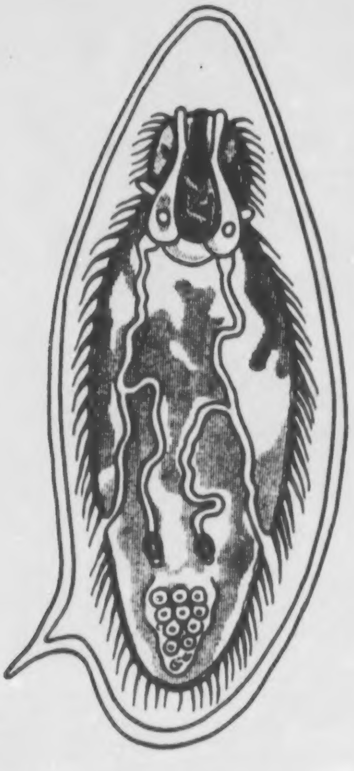


Figure 24. **Egg of *Schistosoma mansoni*.**

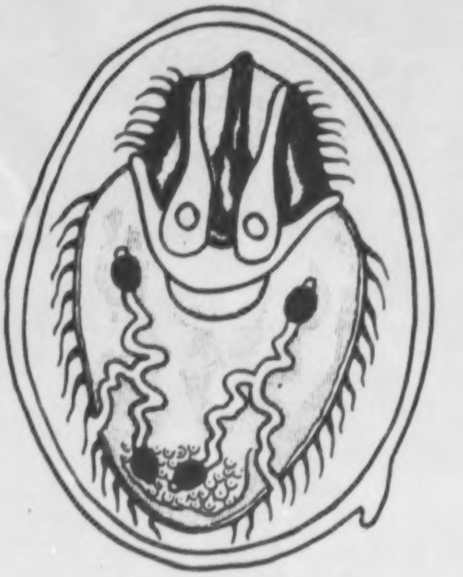


Figure 25. Egg of *Schistosoma japonicum.*

The first two stages comprise the acute stage of the disease. The period of proliferation and tissue repair is known as the chronic stage. During the chronic stage, the liver and spleen enlarge greatly. Intestinal disturbances during the chronic stage are caused by thickening of the intestinal wall, the formation of ulcers, and the development of polypoid growths.

7-14. You can assist in making a specific diagnosis of this disease by finding the characteristic eggs of *S. japonicum* in fecal specimens. You will find the methods recommended for detecting the eggs of *S. mansoni* satisfactory for detecting those of *S. japonicum.*

7-15. *Schistosoma haematobium.* This parasite is also known as the vesical blood fluke. The disease caused by *S. haematobium* is sometimes called vesical schistosomiasis, schistosomiasis haematobia, urinary bitharziasis, or schistosomal hematuria, depending upon the geographical location where the cases are found. The disease is widespread in Africa and surrounding islands. Occasional cases have been found in the southern tips of Europe and India.

7-16. The life cycle of *S. haematobium* is very similar to that of *S. mansoni* and *S. japonicum.* However, the coupled worms usually inhabit the finer veins of the bladder and uterus rather than the mesentery of the intestine as with the other two species. On rare occasions, however, they do lodge in the venules of the intestinal mesentery.

7-17. Man is the only definitive host for *S. haematobium,* and snails of the Genus *Bulinus* are the most common intermediate hosts. Morphologically, *S. haematobium* closely resembles *S. mansoni;* however, adult *S. haematobium* are slightly larger than *S. mansoni.* The male *S. haematobium* has only four or five testes, whereas *S. mansoni* has six to nine testes. The male is covered with minute integumentary tuberosities; but in the female, the tuberosities are usually confined to the extremities.

7-18. The eggs (fig. 26) are 112 to 170 microns long by 40 to 70 microns wide. They are spindle shaped, rounded anteriorly, and conical at the posterior extremity. The posterior end terminates in a blunt-pointed spine. The egg is non-operculate, has a thin transparent shell, and is yellow brown in color. When passed in feces, the egg normally contains a fully developed miracidium.

7-19. The pathological effects of *S. haematobium* can be serious. Penetration of the skin by the cercariae of *S. hematobium* produces almost no local cellular reaction. The pathogenic effects of *S. haematobium* consist of:

a. Local as well as systemic reactions to metabolic products of growing and mature worms.

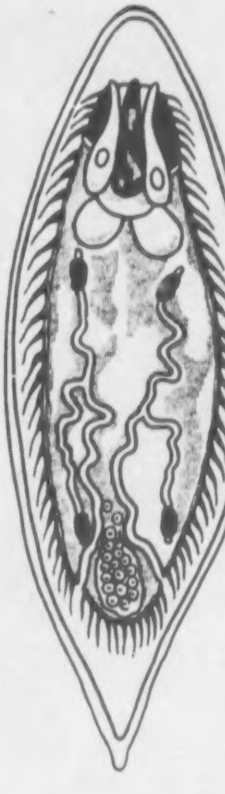


Figure 26. Egg of *Schistosoma haematobium.*

b. Trauma with hemorrhage as eggs escape from the venules.

c. Pseudo-abscess and pseudo-tubercule formation around eggs lodged in the tissues.

Frequently, the only symptom noted in recent infections is bloody urine. Chronic cystitis is often encountered in older infections. Advanced cases of vesical schistosomiasis often result in abscesses, scarring of the bladder wall, and invasion of other body organs.

7-20. Examine both feces and urine for eggs when *S. haematobium* is suspected. The techniques used to examine feces in cases of *S. mansoni* and *S. japonicum* are also satisfactory for *S. haematobium*. Examine urine for eggs by pouring the specimen into a conical flask and allowing it to sediment. Make a smear of the sediment and examine it with the microscope. If a centrifuge is handy, use it to speed sedimentation of the specimen.

7-21. **Liver Flukes.** Liver flukes live in the bile ducts and frequently cause considerable liver damage. None of the liver flukes are found exclusively in man. The two species of greatest general interest are *Fasciola hepatica*, the sheep liver fluke, and *Clonorchis sinensis*, the oriental liver fluke. However, interest in other species discussed in this section is increasing rapidly as more is learned about them.

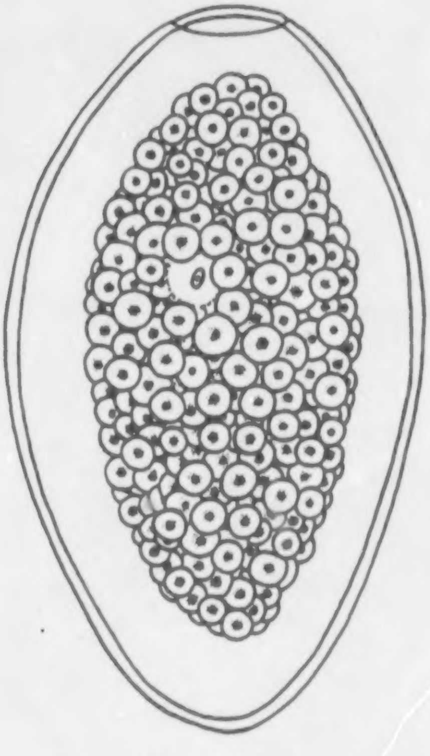


Figure 27. Egg of *Fasciola hepatica*.

7-22. *Fasciola hepatica.* The sheep liver fluke is found in all sheep-raising countries, including the United States. It is known as the "sheep liver fluke" because it commonly infects sheep. Although the parasite is generally associated with sheep and cattle, man becomes infected by ingesting the infective larvae which have encysted on plants such as lettuce and watercress.

7-23. Humans, sheep, and cattle are the definitive host, while snails belonging to the Family Lymnaeidae are the intermediate hosts. Undeveloped eggs are passed in the feces of the definitive host. The eggs mature in 9 to 15 days after reaching water and then free-swimming miracidia escape from the egg shells and penetrate the proper snails. Within the snails the miracidia transform into sporocysts, and the sporocysts produce first-generation and second-generation rediae in about 4 weeks. The rediae produce cercariae, which escape from the snails and encyst on aquatic vegetation as infective metacercariae. After ingestion of contaminated plants by one of the definitive hosts, the metacercariae excyst in the intestine and migrate through the intestinal wall into the body cavity of the infected host. From there they penetrate through the liver parenchyma into the biliary passages, where they develop into mature worms. This migration and development require 3 to 4 months.

7-24. As the metacercariae invade the liver tissues, they produce traumatic and necrotic lesions. Moderate to heavy infections in man give rise to severe symptoms. Patients have hepatic colic, eosinophilia, diarrhea, and eventually anemia. In some human infections, immature as well as mature worms have been found in abscesses in blood vessels, lungs, and even the brain.

7-25. The egg (fig. 27) is one of the largest produced by a helminth that infects man. It is oval in shape and measures about 80 by 150 microns. The egg is yellow brown and has an operculum.

7-26. The usual laboratory examination requires that you identify eggs which you find in feces. Eggs of *F. hepatica* are impossible to differentiate from those of *Fasciolopsis buski;* therefore, you should report them as "eggs morphologically resembling the Genus *Fasciola* and *Fasciolopsis.*" The only way for you to positively identify the parasite as *F. hepatica* is to recover the typical *F. hepatica* eggs from uncontaminated bile collected by the attending physician. This will give you a positive identification, because *F. buski* inhabits the intestine and not the bile ducts.

7-27. False fascioliasis occurs when someone ingests liver infected with *F. hepatica*. The eggs

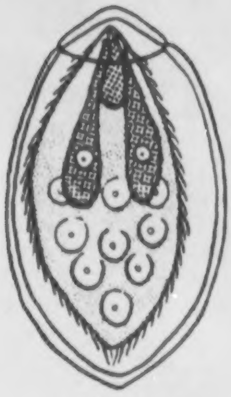


Figure 28. Egg of *Dicrocoelium dendriticum.*

are then passed in the feces of the person eating the liver. You can see how this could easily be mistaken for actual infection. This may be ruled out if the patient is kept on a liver-free diet for 3 days or more. If the patient continues to pass eggs, he probably has a true infection.

7-28. *Dicrocoelium dendriticum. D. dendriticum* is a parasite commonly found in the bile duct of sheep and other herbivorous animals. The parasite is widely distributed in Europe, North Africa, Northern Asia, and some other areas in the Orient. *D. dendriticum* has frequently been reported when actually the patient was not infected at all. This happens quite often when people eat sheep liver that has been infected and the eggs are then passed in the feces just as in false fascioliasis. However, the parasite does infect man and is frequently reported from Europe, Asia, and Africa.

7-29. Snails are the first intermediate host, and ants are the second intermediate host. Man is infected by accidentally ingesting ants that contain infective metacercariae. The eggs (fig. 28) are asymmetrically ovoidal, thick shelled, dark brown in color, have a broad convex operculum, and measure 38 to 45 microns by 22 to 30 microns. They contain a mature miracidium when passed in the feces of the definitive host.

7-30. *Clonorchis sinensis. C. sinensis* is known as the Chinese liver fluke. There are several authors who feel that the genus *Clonorchis* has characteristics sufficiently like *Opisthorchis* to classify the species *sinensis* under the latter genus. Therefore, you may see the term *"Opisthorchis sinensis"* used in some references. This is the most important liver parasite of man in parts of China, Japan, Formosa, and Indochina. There is no evidence that the infection has become established in any region outside the China Sea area. Frozen fish and dried or pickled fish shipped from endemic areas probably account for infection in persons who have never visited areas where the parasite is found. There are many reservoir hosts for this parasite, including the dog and cat.

7-31. The adult worm lives in the bile passages and occasionally in the pancreatic duct. It is a flat, transparent, flabby worm that is somewhat spade shaped. It is relatively small (10 to 25 mm. in length by 3 to 5 mm. in width). One of the most characteristic features of the worm are the large branched testes which are situated one behind the other in the posterior third of the body.

7-32. The eggs are fully embryonated when discharged into the bile ducts. They are passed in the feces, and a miracidium hatches only after the egg is ingested by a snail of the Family Amnicolidae. The miracidium develops a first generation sporocyst in which second generation rediae are produced. Cercariae are produced in the rediae, and they escape from the snail and encyst as metacercariae in the skin of fresh water fish. Man becomes infected by ingesting infected fish. The metacercariae excyst in the intestine and make their way into the common bile duct and finally to the distal bile capillaries, where they develop into mature worms in about 1 month. Approximately 3 months are required for the whole cycle.

7-33. The parasites cause inflammation of the bile ducts, and the body attempts to encapsulate them. This results in the production of considerable fibrous tissue in the bile ducts. In very heavy infections, severe damage may result in cirrhosis of the liver or even death.

7-34. Laboratory diagnosis is based on the recovery of typical eggs from feces. The egg (fig. 29) is small and ovoid with a distinct operculum that fits into a rimmed extension of the shell. They have moderately thick, light yellow-brown shells; and they measure about 29 microns by 16 microns.

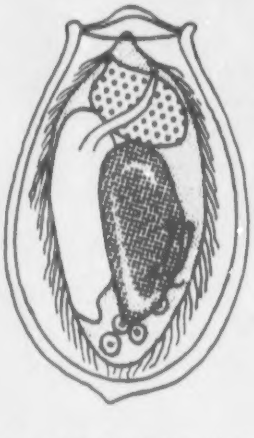


Figure 29. Egg of *Clonorchis sinensis.*

341

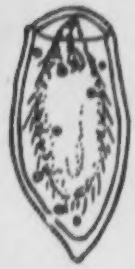


Figure 30. Egg of *Opisthorchis felineus*.

7-35. *Opisthorchis felineus*. *O. felineus* is widely distributed in eastern and southeastern Europe and Asiatic U.S.S.R., and it is reported to be common in Vietnam. The worm is smaller than *Clonorchis* (10 mm. by 2.5 mm. as compared with 20 mm. by 4 mm.); otherwise, they are very similar. The eggs of the two worms are also very similar. They are slightly narrower than those of *Clonorchis*. The egg (fig. 30) of *O. felineus* is 30 microns by 11 microns, whereas that of *C. sinensis* is about 29 microns by 16 microns. A snail is the first intermediate host, and cyprinid fresh water fish are the second intermediate host. In addition to man, many fish eating mammals are infected. Clinical aspects and diagnostic procedures are essentially the same as those of clonorchiasis.

7-36. *Opisthorchis viverrini*. *O. viverrini* is closely related to *O. felineus*. As many as 75 percent of the people from some areas of northeastern Thailand have been found to be infected with the parasite. The eggs are slightly smaller than those of *Clonorchis* and *O. felineus*. They measure 26 microns by 13 microns.

7-37. With our commitments in Southeast Asia, it is important for you to be familiar with this parasite. However, be aware that only the most expert technician can differentiate the eggs of *Clonorchis*, *Opisthorchis*, and *Metagonimus*, an intestinal fluke, which is discussed later in this section.

7-38. **Intestinal Flukes.** In this country, intestinal flukes are of little medical importance. There are no known intestinal flukes that are strictly human parasites. A number of species parasitize humans as well as other animals. In certain localities, this condition constitutes a serious medical problem because the other animals form a large reservoir of infection. The best example of this is *Fasciolopsis buski* in southeastern Asia, where it is a common parasite of pigs and humans.

7-39. *Fasciolopsis buski*. This fluke is called the giant intestinal fluke. It is most common in India, China, and the southwest Pacific. It is found in man, swine, and occasionally in dogs.

7-40. *Fasciolopsis buski* is the largest and best known of the intestinal flukes of man. It measures over an inch in length and about a half inch in width. The worm is leaf-like in appearance and has a spinose integument. The ventral sucker (acetabulum), located near the anterior end, is three to four times as large as the oral sucker. *F. buski* has highly branched testes, which occupy the posterior three-fifths of the body.

7-41. The eggs must get into fresh water after being passed in feces. There they hatch in 3 to 7 weeks. A miracidium escapes from the egg and penetrates an appropriate snail intermediate host of the Genus *Hippeutis* or *Segmentina*. After the miracidium undergoes the necessary development, cercariae escape from the snail and encyst on water chestnut or water caltrop as metacercariae. Human infection occurs when a person ingests the metacercariae while cracking nuts of the above plants with his teeth.

7-42. The egg is practically identical to that of *F. hepatica*, shown in figure 27. It is operculate and measures 130 microns to 140 microns by 80 microns to 85 microns. Diagnosis is based on the recovery of typical eggs from feces and on the physicians's clinical findings.

7-43. Pathological effects of *F. buski* include anemia, emaciation, and inflammation and ulceration of the intestinal mucosa. Intestinal obstruction and toxic reactions frequently occur in cases of heavy infection.

7-44. *Heterophyes heterophyes*. *H. heterophyes* is found in Egypt, Palestine, and the Orient. Suitable hosts include dogs, cats, rabbits, and a few other mammals, as well as man. Snails of the Genus *Pironella* are first intermediate hosts, and fresh water fish are second intermediate hosts.

7-45. Adult parasites live in the small intestine of the definitive host. Frequently they are present in large numbers. The eggs are completely embryonated when they pass from the host. Miracidia hatch from the eggs after the eggs are ingested by the proper snail. After the necessary development is completed in the snail, cercariae escape from the snail and penetrate beneath scales of certain fish, where they encyst as the infective metacercariae. The final host becomes infected by eating uncooked fish that contain metacercariae.

7-46. The adult worm is very small. It measures approximately 0.4 mm. by 1.5 mm. The oral sucker is ventrally located, but it is only about one-third as large as the acetabulum. The adult worm is covered with minute spines which are set closely together.

7-47. The egg (fig. 31) is small (30 microns by 17 microns), operculate, brownish in color, and contains a well-developed ciliated miracidium.

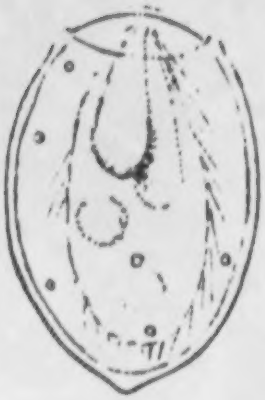


Figure 31. Egg of *Heterophyes heterophyes.*

It will be very difficult for you to differentiate the eggs of this parasite from *Opisthorchis, C. sinensis,* or *M. yokogawai.* You can easily recover the eggs from feces by the standard procedures.

7-48. *H. heterophyes* attaches itself to the intestinal mucosa, provoking symptoms of mild diarrhea, usually with no serious pathology. Occasionally the worms bore their way through the mucous lining of the intestine. When that happens, the minute eggs penetrate into the mesenetric lymphatics. They have been known to reach the heart and promote cardiac failure.

7-49. *Metagonimus yokogawai. M. yokogawai* is a common parasite of the Far East and the Balkan States. Man and several other mammals are suitable definitive hosts. Snails of several genera serve as the first intermediate host, and certain fishes act as the second intermediate host. Man becomes infected by eating uncooked freshwater fish on which the infective metacercariae have encysted.

7-50. The infective metacercariae of *M. yokogawai* excyst in the small intestine of the definitive host and attach themselves to cells in the mucosal crypts and grow to maturity. Normally the eggs are passed into the intestinal lumen, and pass out with feces. However, some of the worms may penetrate so deeply into the intestinal wall that some eggs can get into the general blood circulation, as in *H. heterophyes* infections.

7-51. The egg (fig. 32) is operculate, brownish in color, measures 28 microns by 17 microns, and contains a well developed ciliated miracidium. Diagnosis is based on the recovery of characteristic heterophyid eggs in feces. More than likely you will not be able to differentiate the eggs of *M. yokogawai* from those of *H. heterophys,* and you can expect to have difficulty in differentiating them from those of *Clonorchis* and *Opisthorchis.* Experts differentiate them by extensive study of morphological differences and also study of life-cycle forms.

7-52. Patients with *M. yokogawai* usually have persistent intestinal disturbances and diarrhea. Eggs that get into the circulating blood may reach the heart and central nervous system, where they can cause serious cardiac and nervous disturbances.

7-53 **Lung Fluke (Paragonimus westermani).** The only lung fluke of man is *Paragonimus westermani.* It is a common infection of man in Asia. A very similar or identical fluke is found in cats, dogs, pigs, and wild carnivores in Africa and North and South America.

7-54. Humans are commonly infected with this species in Japan, Korea, Formosa, the Philippines, and Thailand. It is endemic in small foci of Africa and northern South America. *P. Westermani* has been recovered from wild felines in India, Malaysia, Indonesia, and Thailand.

7-55. Morphologically the adult is plump and ovoidal in cross section, about 12 mm. long and one-third as wide. The worm is reddish brown when alive. Its surface is covered with large spines.

7-56. The adult worms usually live in pairs in the parenchyma of the lungs. Eggs are deposited around the worms, and many of them are able to get into the respiratory passages. They are coughed up, swallowed and pass from the host in feces. The eggs are unembryonated and require approximately 16 days in clear running water to complete their development. Then the eggs hatch, releasing a miracidium which penetrates a snail intermediate host. The miracidium transforms into a sporocyst, and within the sporocyst many rediae develop. Each rediae produces many cercariae which escape from the snail and penetrate suitable freshwater crabs and crayfish. The cercariae then

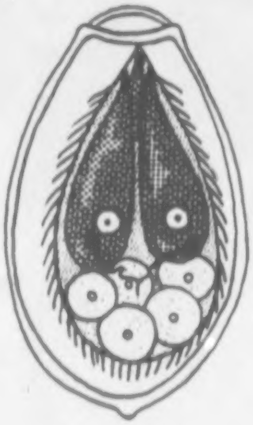


Figure 32. Egg of *Metagonimus yokogawai.*

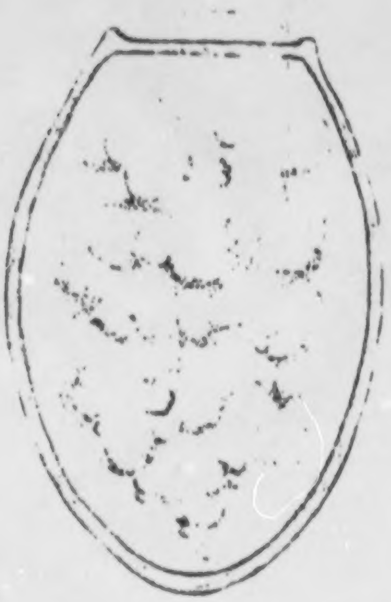


Figure 33. Egg of *Paragonimus westermani.*

encyst as infective metacercariae. When the infected crabs or crayfish are eaten raw, the metacercariae are freed in the small intestine. They burrow through the intestine into the peritoneal cavity, then through the diaphragm and on into the lungs.

7-57. The eggs of *P. westermani* (fig. 33) are relatively large, measuring approximately 80 to 120 microns in length by 45 to 60 microns in width. The egg shell is relatively thick and golden brown in color. The eggs have a flat-shaped operculum, and they are unembryonated when passed.

7-58. A *Paragonimus* infection causes a chronic bronchical cough with bloody sputum. The bloody sputum is the result of small blood vessels in the capsule around the worms leaking into a bronchiole, thus allowing blood and eggs to get into the sputum. The worms do not always find their way to the lungs. They have been found in many other organs. Frequently in these cases, abscesses are formed and death may result.

7-59. Specific diagnosis is easily made when the eggs of *P. westermani* are recovered from rusty or blood-tinged sputum, feces, pleural aspirates, or from peritoneal abscesses. In suspected cases where eggs cannot be recovered, intradermal tests and complement fixation tests may prove to be very helpful.

8. Cestodes (Tapeworms)

8-1. Adult tapeworms are flat, ribbon-like, segmented worms. The individual segments are called *proglottids*. The worm does not have a mouth, gut, or body cavity. They attach themselves to the intestinal mucosa by a scolex (head) that has suckers. The adult worm (fig. 34) is separated into the following recognizable regions: (a) scolex, (b)

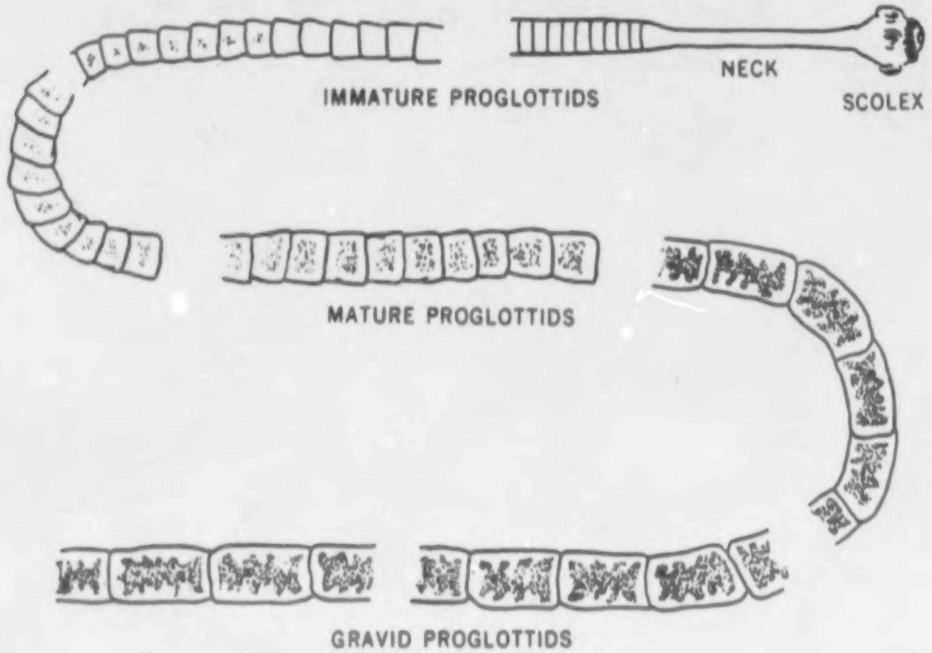


Figure 34. Adult tapeworm.

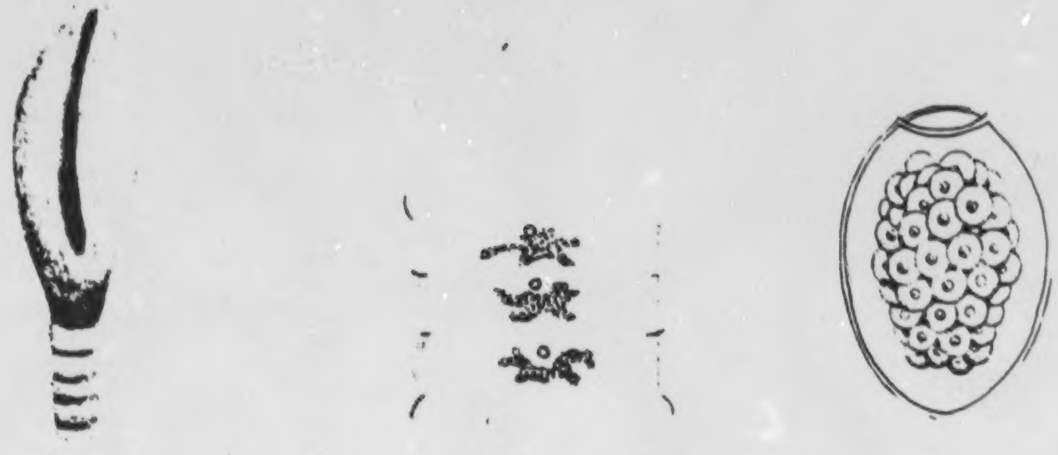

SCOLEX PROGLOTTID EGG

Figure 35. *Diphyllobothrium latum.*

neck, a region of growth that immediately follows the scolex, (c) immature proglottids, (d) mature proglottids, and (e) gravid proglottids. The entire length of the worm that is made up of proglottids is called the *strobila.*

8-2. The scolex of *Diphyllobothrium latum* is long and spatulate with a long, sucking groove on each side. The other tapeworms that infect man have knob-like scolices which possess cup-like suckers at each of the four angles. Some of them also have an anterior muscular projection *(rostellum)*

8-3. There is a complete set of male and female organs for each mature proglottid. *Diphyllobothrium* discharges *unembryonated* eggs that must reach cool, clear water before further development occurs. The eggs of the other cestodes contain *fully developed* hexacanth embryos which have three pairs of hooklets.

8-4. The larval stages of some cestodes can parasitize man. In such infections, the larvae may be found in almost any tissue of the body. In cases of larval infection by cestodes such as *Echinococcus,* the infection can be fatal. In some geographical locations human infections with the larval cestodes are quite common. The diagnosis of these larval infections is usually made by a pathologist from examination of histological sections of biopsy material.

8-5. As a means of getting eggs out of the host, some cestodes discharge eggs singly, some shed one entire gravid proglottid at a time, and some shed groups of gravid proglottids. Recovery and identification of the scolex is necessary for treatment to be considered satisfactory. The six species listed in table 4 are the cestodes that commonly parasitize man. These parasites live as adults in the intestine of man. Each of the species that frequently infect man will be discussed in this section.

8-6. **Diphyllobothrium Latum.** This parasite is commonly known as the fish tapeworm or broad tapeworm. The worm may be up to 35 feet long and may have as many as 4,000 proglottids. *D. latum* is a common parasite in parts of Europe, Russia, Japan, Philippines, Australia, South America, and North America. Dogs, wolves, bears, and a few other carnivores in addition to man are hosts for this worm.

8-7. The eggs are passed in feces and require about 2 weeks in cool, clear water to complete embryonation. When the egg hatches, a coracidium (ciliated embryo) escapes through the operculum and swims about. The coracidium must be eaten by a small crustacean within 12 hours. In the crustacean the coracidium develops into a *procercoid* larva. The crustacean infected with the procercoid larva must be eaten by a freshwater fish before larval development can be completed. In the fish, the procercoid larva migrates to muscle tissue and develops into a *plerocercoid* larva *(sparganum).* When an uncooked fish containing a *plerocercoid* larva is ingested by man or some other suitable carnivore, the larva attaches to the intestine, where it matures and passes eggs, thus completing the cycle.

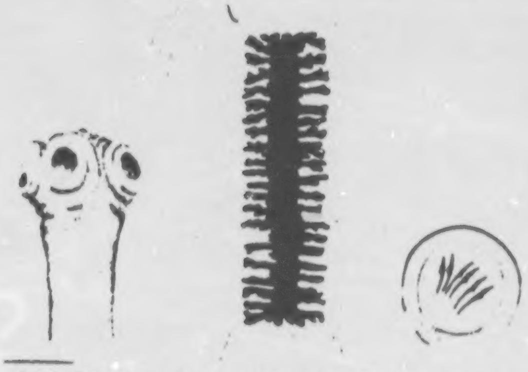


Figure 36. *Taenia saginata.*

8-8. The scolex (fig. 35) of an adult *D. latum* is small and spoon-shaped. It has two longitudinal slits *(bothria),* which serve as sucking organs for attachment. Neither hooks nor true suckers are present. The mature proglottids of *D. latum* (fig. 35) are broader than they are long, whereas the gravid proglottids are about as long as they are wide. The principal diagnostic features of mature and gravid proglottids are the centrally situated rosette-shaped uterus and a genital pore which lies in the center of each segment. *D. latum* eggs (fig. 35) are broadly ovoid and shaped very much like a hen egg. The shell is moderately thick and golden-brown when passed in feces. The eggs vary greatly in size (59 microns to 71 microns by 42 microns to 49 microns), and they contain a partially developed embryo when passed. At the anterior pole of the egg there is a prominent operculum, and at the posterior pole there is a small knob-like protuberance.

8-9. The symptoms produced in man by *D. latum* are nausea, loss of appetite, abdominal discomfort, weakness, hunger, weight loss, and anemia. Occasionally, infected individuals may vomit portions of a worm. You should not have any difficulty in properly identifying the worm, because the characteristics of the scolex, proglottids, and the eggs are all very specific for the species.

8-10. **Taenia Saginata.** This is the largest tapeworm that infects man. The worm is usually about 15 feet long, but it may reach a total of 75 feet. There are usually 1,000 to 2,000 proglottids. *T. saginata* is widely known as the beef tapeworm. It is distributed worldwide, but is most prevalent in countries of southwestern Europe, Africa, and South America.

8-11. Man is the definitive host for the adult worm, and cattle are the intermediate hosts. The proglottids and eggs are scattered on soil and grass through human feces. When the embryonated egg is ingested by a cow, the hexacanth embryo hatches from the egg in the cow's intestine. The embryo bores through the intestinal wall and gets into the blood or lymph circulation. The embryo is carried to the cow's skeletal muscles, where it encyst and develops into a typical cysticercus larva in 60 to 75 days.

8-12. The scolex (fig. 36) of *T. saginata* has no rostellum nor attachment hooks. The attachment organs consist of four cup-shaped hemispherical suckers, one at each of the four corners of the scolex. Mature proglottids are slightly broader than long, whereas the gravid proglottids are considerably more narrow and about three times as long as the broadest portion of the segment. The central tube of the uterus of a gravid proglottid (fig. 36) has 15 to 20 lateral branches on each side. The entire uterus is filled with thick shelled eggs. The eggs (fig. 36) are longitudinally striated. The striations are very fine and close together, and they are readily observed with a microscope using reduced light. There is a clear line of demarcation around the hexacanth embryo, since the embryo does not completely fill the egg.

8-13. *T. saginata* is often the cause of intestinal problems because of its large size. It causes appendicitis, systemic intoxication, hunger pains, diarrhea, vomiting, and weight loss. It can also cause intestinal obstruction. The infection can mimic the symptoms of peptic ulcer and gallbladder disease.

8-14. You cannot differentiate *T. saginata* from the pork tapeworm *(T. solium)* solely on the basis of egg morphology. You should report that eggs of the Genus *Taenia* are present. You can make a positive identification of *T. saginata* from a gravid proglottid. A gravid proglottid of *T. saginata* has 15 to 20 main lateral branches on each side of the uterine stem, whereas *T. solium* has only 7 to 13 uterine branchs. Press a gravid proglottid between two glass slides and then count the lateral branches with the use of a hand lens.

8-15. **Taenia Solium.** *T. solium* is found in areas where people eat improperly cooked pork. Man is the only definitive host for the adult worm, and hogs are the usual intermediate hosts. Naturally, the best way to control the parasite is to cook all pork and to treat all human cases.

8-16. Gravid proglottids are passed in human feces, and the eggs are discharged from the proglottids when they reach the soil. The eggs must be ingested by a pig or a man for development to progress. After a man or a pig ingests an egg, a hexacanth (six-hooked) embryo hatches from the egg and bores through the intestinal wall and is carried by the blood or lymph circulation to various tissues of the body. The embryo develops into a cysticercus larva within 2 to 3 months. A larval

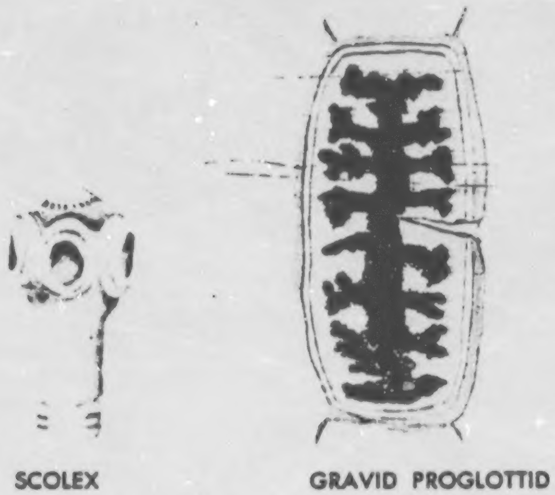


Figure 37. *Taenia solium.*

infection is known as cysticercosis. The cysticercus is the infective stage that leads to development of the adult *Taenia solium*. The adult tapeworm develops *only* after man ingests raw or improperly cooked pork which contains an infective cysticercus larva.

8-17. The scolex (fig. 37) of the adult *T. solium* has four suckers and a prominent rounded rostellum that is armed with a double circle of hooks. There are 22 to 32 hooks, alternately large and small in a regular pattern on the margin of the rostellum. Mature proglottids are slightly wider than long. The elongated, egg-filled, gravid proglottid (fig. 37) has a characteristic uterine branching pattern which is diagnostic for this species of *Taenia*. The uterus of *T. solium* consists of a single longitudinal, centrally situated tube which has 7 to 13 (usually 9) branches on each side. The eggs cannot be distinguished from those of *Taenia saginata*. You can easily identify *T. solium* by examining the scolex or a pressed gravid proglottid, using the method discussed in the section on *T. saginata*.

8-18. *T. solium* infection causes irritation of the mucosa of the intestine and on rare occasions causes obstruction of the intestine. The infection may result in nervous disorders due to the production of toxic substances by the adult worm. In cases of cysticercosis, the larvae are most frequently located in subcutaneous tissues; but they have been found in practically every organ and tissue of the body. When they lodge in the brain, the outcome is often fatal.

8-19. **Hymenolepsis Nana.** *H. nana* is found all over the world, but it is most prevalent in warm climates. This worm is known as the dwarf tapeworm of man; however, rats and mice are also definitive hosts. It is common in Europe, Russia, Latin America, India, and the southern United States. Of all the tapeworms found in America, *H. nana* is the most common. The worm is only about 2 inches long but can have as many as 200 proglottids.

8-20. The eggs are embryonated when passed. *H. nana* does not require an intermediate host. When eggs are ingested by a definitive host, a hexacanth embryo is liberated in the small intestine. The embryos bore into the intestinal mucosa, where they transform into cysticercoid larvae in about 1 week. The cysticercoid larvae migrate back into the intestinal lumen, attach to the mucosa and develop into mature worms in about 2 weeks. In some cases, the eggs of *H. nana* may

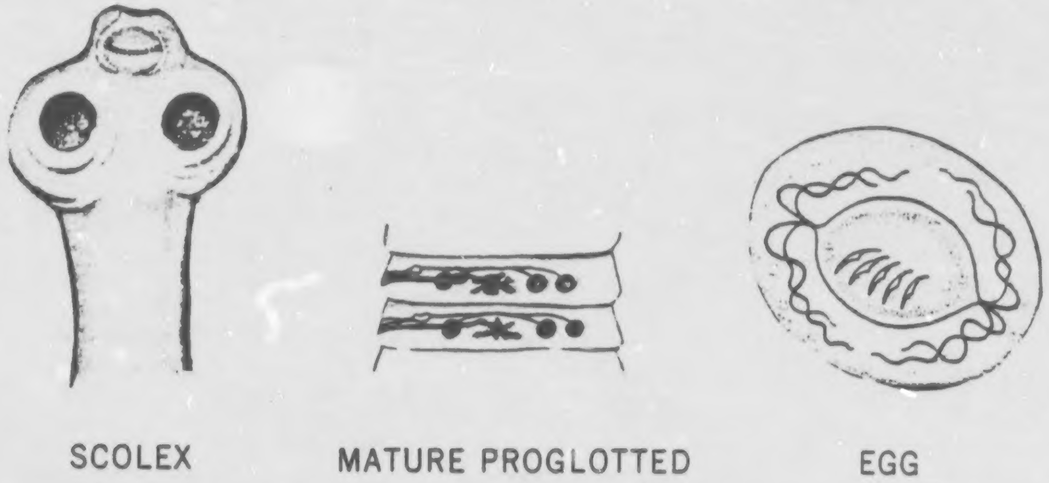
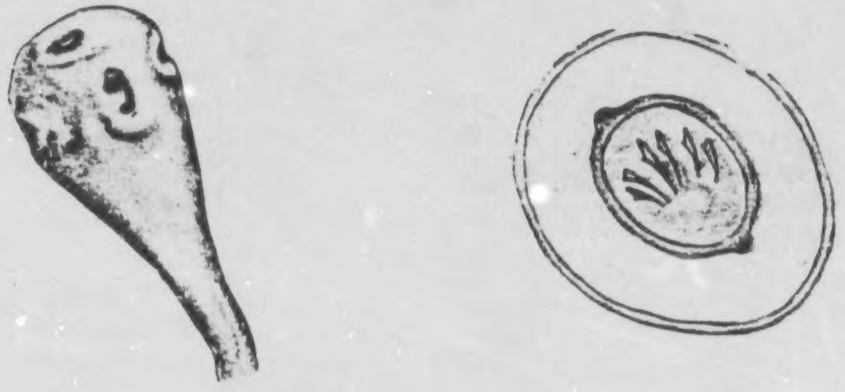


Figure 38. *Hymenolepis nana.*



SCOLEX EGG

Figure 39. *Hymenolepis dimunuta.*

hatch as they travel down the intestine. This phenomenon is known as internal autoinfection and is thought to be responsible for some of the very heavy infections that are frequently encountered.

8-21. Recognition of the tapeworm is based on the following characteristics:

- The scolex (fig. 38) has four suckers, and a rostellum that is armed with a single row of 20 to 30 hooklets.
- The mature proglottids (fig. 38) are much broader than long, and there are three testes present that are arranged in a row across the proglottid.
- The eggs (fig. 38) are nearly spherical and measure 30 to 47 microns in diameter. The egg shell is made up of two thin membranes; the inner membrane has polar knobs to which threadlike filaments are attached. The eggs contain hexacanth embryos when passed.

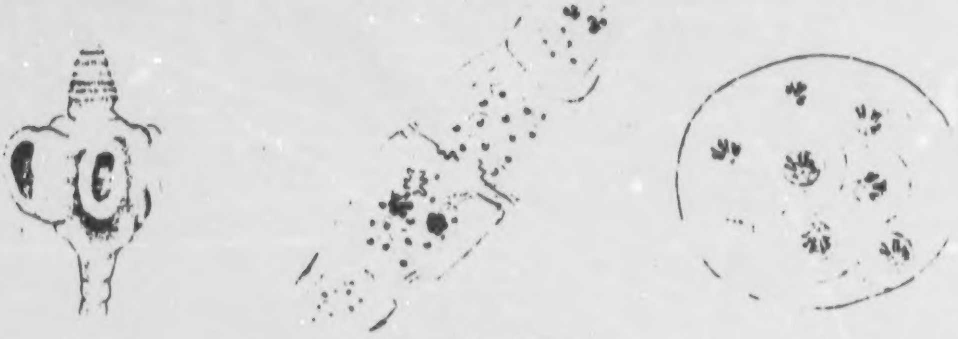
8-22. Light infections may or may not produce symptoms. There may be diarrhea, vomiting, insomnia, weight loss, and even allergic reactions. Heavy infections almost always produce moderate to profuse diarrhea, nervous disorders, abdominal pain, and in some cases, extreme apathy.

8-23. You will be expected to identify the characteristic eggs of *H. nana*. When the eggs are present, you should look for the characteristic polar knobs and filaments. When only worms are available, stain the mature proglottids. The presence of three ovoid testes along the lower margin is characteristic for the genus *Hymenolepsis*. When eggs are not available, you should examine the scolex of an adult worm to differentiate *H. nana* from the rat tapeworm, *H. diminuta*. The scolex of *H. nana* has a rostellar crown of 20 to 30 hooklets which may be inverted, whereas the scolex of *H. diminuta* has a deep sucker-like pocket into which the small rostellum is usually retracted. The rostellum of *H. diminuta* does not have hooklets.

8-24. **Hymenolepsis Diminuta.** This tapeworm is a common parasite of rats, mice, and other rodents; therefore, it has been given the name rat tapeworm. However, it is occasionally found in humans, usually children. *H. diminuta* has been reported from human hosts from most areas of the world.

8-25. *H. diminuta* is small in comparison with some of the other tapeworms. Nevertheless, it is considerably larger than *H. nana*. It may be 20 inches long and have up to 2,000 proglottids. As you can see in figure 39, the scolex of *H. diminuta* is knob-like and has four relatively small suckers. At the tip of the scolex you can see a deep sucker-like pocket into which a small, unarmed rostellum is retracted. The mature proglottids are about three times as wide as they are long. Each mature proglottid contains three ovoid testes in a line across the proglottid. The gravid proglottids disintegrate, releasing fully embryonated eggs. The eggs, as illustrated in figure 39, are similar to those of *H. nana;* but with a little study, you will have no difficulty telling them apart. The eggs of *H. diminuta* are larger, 72 to 86 microns by 60 to 79 microns; and the polar filaments which are characteristic of *H. nana* are not present.

8-26. After the eggs are voided in feces, they must be ingested by a suitable arthropod before further development is possible. The arthropods in this case are larval rodent fleas, meal moths, and many species of beetles. Cysticercoid larvae develop in the required intermediate hosts. When the arthropod intermediate host is ingested, the cysticercoid larvae is freed and attachs itself to the in-



SCOLEX GRAVID PROGLOTTID EGG

Figure 40. *Dipylidium caninum.*

testinal mucosa. Complete development in the definitive host requires about 3 weeks.

8-27. *H. diminuta* is similar to *H. nana* in many ways. The worms are morphologically similar, and the symptoms they produce are much the same. You can differentiate the eggs of *H. diminuta* from those of *H. nana* because the eggs of the former have no polar filaments. Remember that you will have to use sedimentation or centrifugation techniques and not flotation methods to concentrate the eggs. The methods for identifying the whole worm when eggs are not available are the same as those discussed in the section on *H. nana.*

8-28. **Dipylidium Caninum.** This is a common parasite of both the dog and cat, and it is an occasional parasite of humans. Children are most often infected because childen are frequently in close contact with infected pets. This parasite has a worldwide distribution and is known as the dog tapeworm.

8-29. The scolex (fig. 40) of *D. caninum* has four rounded suckers and a retractile rostellum with six rows of minute hooklets. *D. caninum* is about the same size as *H. diminuta* but has fewer proglottids. Both worms are about 20 inches long, but *D. caninum* usually has fewer than 1,000 proglottids, whereas *H. diminuta* has between 1,000 and 2,000. The proglottids of *H. diminuta* are very wide from side to side and narrow from top to bottom, but those of *D. caninum* are long and slender. The gravid proglottids of *D. caninum* (fig. 40) are shaped much like cucumber or pumpkin seeds. This is the only tapeworm infecting man that has proglottids with genital pores on each side. The intact gravid proglottids contain many polygonal-shaped egg capsules (fig. 40) which contain numerous embryonated eggs. The ripe proglottids separate from the strobila and pass from the host. Upon reaching the soil, they fragment, setting free the egg capsules. The individual eggs are almost spherical, 45 microns x 50 microns, with a shell of two thin layers.

8-30. The expelled eggs must be ingested by the dog louse or larvae of certain fleas before further development can occur. The eggs hatch in the arthropods, and the embryos transform into cysticercoid larva. The definitive host becomes infected when an infected arthropod intermediate host is ingested. After the infected arthropod is ingested, the cysticercoid larva attaches to the intestinal mucosa of the definitive host and develops to a mature worm.

8-31. *D. caninum* may cause profuse diarrhea and unrest in children. In some cases the worm may cause sensitization reactions such as urticaria, fever, and eosinophilia. You will have no difficulty in making the proper identification if you find any of the characteristic parts of the tapeworm.

CHAPTER 4

Nematodes Infecting Man

THE UNSEGMENTED roundworms make up the Phylum Nematoda. Members of this group have a complete digestive tract. They have a body cavity, but it is not lined with mesothelium like a true body cavity. It is usually packed with reproductive organs. The sexes are usually separate.

2. While many species of nematodes are free living, many parasitize plants and many others are obligate parasites of animals. Nematodes are the most common parasites of man. Among native populations in tropical countries where sanitation is poor, nematode infections are universal. Even though these parasites are not serious problems in the United States today, they are problems for our troops stationed in areas where large reservoirs of infection exist.

3. Nematode life cycles include the following fundamental stages: the egg, four larval stages, and the adult. The number of eggs produced per day varies greatly from one species to another. The eggs of different nematodes are in specific stages of development when passed. This phenomenon ranges from one extreme to the other. Some nematodes pass unembryonated eggs, whereas others pass eggs that are fully embryonated. Some even retain the eggs in-utero until they hatch and then pass the larvae.

4. On the basis of their habitat, nematodes of humans are divided into the intestinal roundworms and the somatic or tissue roundworms. With the exception of *Trichinella*, the intestinal roundworms do not require an intermediate host; they have a more or less direct life cycle. The somatic or tissue roundworms and *Trichinella* require intermediate hosts; they have indirect life cycles.

5. This chapter is divided into three sections. The first section covers the intestinal nematodes of humans. The second section includes the nematodes of humans that live as adults in the tissues. The third section deals with nematodes that normally parasitize other animals but which are capable of entering the human body. Table 5 illustrates the taxonomic relationship of the various members of the Phylum Nematoda discussed in this chapter.

9. Intestinal Nematodes

9-1. The intestinal nematodes, with the exception of *Trichinella*, do not have an intermediate host in their life cycle. The eggs or larvae are passed in feces and require a period of development outside the host to reach the infective stage. The life cycles of the intestinal nematodes vary in complexity from the very simple pattern of *Enterobius* to the involved pattern of *Strongyloides*.

9-2. **Enterobius Vermicularis.** *E. vermicularis*, the pinworm or seatworm, is cosmopolitan in distribution; but it is more common in cool or temperate regions than in strictly tropical areas. It is the most common nematode infecting humans in the United States. It is most frequently found in small children who live in crowded conditions.

9-3. Adults (fig. 41) are cylindrical in shape and whitish in color. As with most nematodes, the male is smaller than the female. The male is about 2 mm. to 5 mm. long, and the female is approximately 10 mm. long. The posterior end of the male is sharply curved so that its body resembles an upside down question mark.

9-4. The eggs of *E. vermicularis* (fig. 41) are more or less flat on one side and broadly rounded on the other. They have a colorless double shell that is very sticky. This sticky outer shell makes the egg stick to clothing and to the perianal skin. The eggs are almost fully embryonated when passed. In a matter of a few hours after passage the eggs are infective.

9-5. Infective eggs are accidentally ingested from contaminated fingers or along with dust. They hatch in the small intestine; and larvae make their way to the large intestine, where they mature in from 15 to 28 days. After mating, the gravid female migrates to the anus and crawls out, whereupon she usually ruptures—spilling eggs all over the perianal folds.

9-6. The presence of worms and eggs on the skin causes anal itching, which is quite severe at

TABLE 5
NEMATODA

- PHYLUM NEMATODA
  - CLASS APHASMIDIA
    - SUPERFAMILY Trichuroidea
      - GENUS *Trichinella*
      - *Trichuris*
      - *Capillaria*
    - SUPERFAMILY Dioctophymatoidea
      - GENUS *Dioctophyma*
  - CLASS PHASMIDIA
    - SUPERFAMILY Rhabdiasoidea
      - GENUS *Strongyloides*
    - SUPERFAMILY Strongyloidea
      - GENUS *Ancylostoma*
      - *Necator*
      - *Ternidens* -
      - *Syngamus*
    - SUPERFAMILY Trichostrongyloidea
      - GENUS *Trichostrongylus*
    - SUPERFAMILY Metastrongyloidea
      - GENUS *Angiostrongylus*
    - SUPERFAMILY Oxyuroidea
      - GENUS *Enterobius*
    - SUPERFAMILY Ascaridoidea
      - GENUS *Ascaris*
      - *Toxocara*
      - *Lagocheilascaris*
      - *Anisakis*
    - SUPERFAMILY Spiruroidea
      - GENUS *Gongylonema*
      - *Gnathostoma*
      - *Physaloptera*
      - *Thelazia*
    - SUPERFAMILY Filarioidea
      - GENUS *Wuchereria*
      - *Brugia*
      - *Onchocerca*
      - *Dipetalonema (=Acanthocheilonema)*
      - *Mansonella*
      - *Dirofilaria*
      - *Loa*
    - SUPERFAMILY Dracunculoidea
      - GENUS *Dracunculus*
- PHYLUM ACANTHOCEPHALA
  - *Macracanthorhynchus*
  - *Moniliformis*

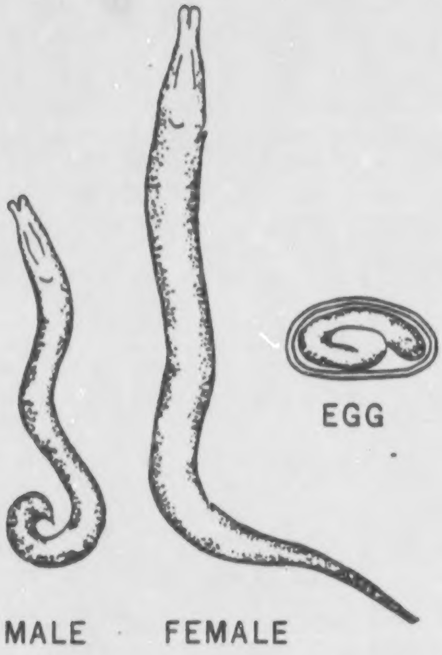


Figure 41. *Enterobius vermicularis.*

times. Occasionally, in female patients, a worm will crawl into the vagina, on into the fallopian tubes, and eventually into the peritoneal cavity, causing severe complications. Pinworms are normally inhabitants of the cecum. Because they are frequently found in the appendix, they are often suspected of causing appendicitis; but their presence is probably incidental.

9-7. Laboratory diagnosis is made by finding typical adults or eggs. Because of the migratory behavior of gravid females, eggs are only rarely found in routine fecal examinations. You should instruct the parents to collect the specimen by pressing the sticky side of *clear* scotch tape to the perianal folds and then sticking the tape to a glass slide. It is best to get the specimen 2 to 4 hours after the patient has gone to bed or early in the morning before bathing and before a bowel movement. Put a few drops of xylene under the tape just before examining the specimen, and most of the confusing air bubbles will be eliminated.

9-8. **Trichuris Trichiura.** *Trichuris* (whipworm) infections are widely distributed, but they are prevalent only in warm or moist temperate climates. The worm is frequently present in individuals who also harbor *Ascaris* and hookworm.

9-9. *Trichuris* eggs, in the unsegmented stage, are passed from the host in feces. They are not infective until 10 to 14 days of development in moist, shady soil. Man becomes infected by ingesting infective eggs. The eggs hatch in the small intestine, and the emerging larvae enter the intestinal crypts and penetrate into the glands and stroma, where they obtain nourishment. They gradually migrate down the intestine; and in about 10 days they begin to appear in the cecum, which is the habitat of the adult worms. The worms reach maturity in about 90 days after the eggs are ingested. These worms usually live for several years.

9-10. The anterior two-thirds of the worm is slender and thread-like, whereas the posterior one-third is thick and fleshy. The anterior of the worm is delicately but firmly threaded into the mucosa of the cecum. The male (fig. 42) measures 30 to 45 mm. in length. Its heavy posterior end is heavy set and curled into a full circle. The female (fig. 42) measures 35 to 50 mm. in length. Its body is bluntly rounded at the posterior end. The female *T. trichiura* has only one ovary and produces relatively few eggs (3,000 to 6,000 per day). The eggs (fig. 42) measure 50 to 54 microns by 22.5 microns. They are barrel shaped and have a double shell. The outer shell is stained with bile, which gives it a golden brown color. They have what appear to be unstained mucoid plugs at each end of the shell.

9-11. Light infections do not usually cause symptoms. The presence of moderate to large numbers of worms causes bloody diarrhea. In direct saline smears from such cases, you will find eggs, many eosinophils, and Charcot-Leyden crystals. The eosinophils and Charcot-Leyden crystals may even be present several weeks before the worms start to produce eggs. You will have to use a concentration technique to find the eggs in light infections. The zinc sulfate centrifugal flotation method is generally excellent, but it will not float

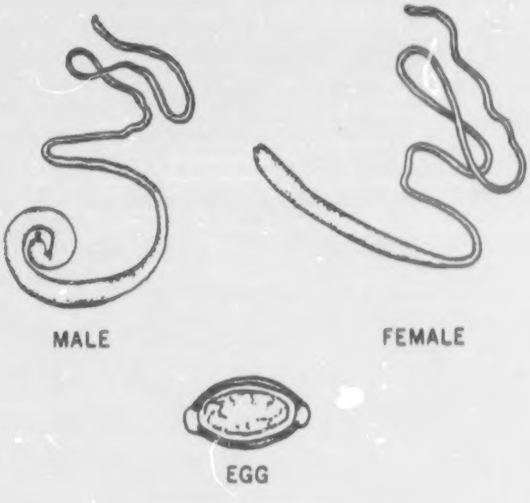


Figure 42. *Trichuris trichiura.*

infertile nematode eggs. The acid-formalin-ether concentration method is satisfactory, and the Kato thick smear has recently been shown to be excellent for detecting nematode eggs.

9-12. **Ascaris Lumbricoides.** *A. lumbricoides* is found all over the world except in areas that are very cold and dry. It is most prevalent in tropical areas, but it is a common parasite in cooler climates as well.

9-13. The eggs of *Ascaris* are unsegmented when passed and must undergo a period of development in the soil before they are infective. Under favorable conditions, the eggs develop to the infective stage in about 3 weeks. The eggs may remain viable in soil for months or even years. They are very resistant to drying and low temperatures.

9-14. Digestive juices act on the egg shell, and the larva escapes in the small intestine. The larva migrates through the intestinal mucosa and makes its way to the liver, then to the heart, and finally to the capillaries of the lungs. There it enters an alveolus, where it undergoes one molt and grows to about 2 mm. in length. After about 9 days in the alveolus, the third stage larva migrates up the trachea and is swallowed. In the intestine two more molts occur, and the worms become sexually mature in 8—12 weeks from the time the eggs were ingested. They usually live for about 1 year.

9-15. Adult *A. lumbricoides* (fig. 43) are large worms. The females measure from 20 to 45 cm. in length and are about 5 mm. in diameter; the males are roughly two-thirds as long and somewhat smaller in diameter. The female produces an average of about 200,000 eggs per day. The characteristic fertile egg (fig. 43) has a thick, transparent inner shell that is covered by an irregular, wrinkled albuminous coat. The fertile egg contains a coarsely granular spherical egg cell which usually does not completely fill the shell. Bile pigments in the intestinal tract of the host stain the eggs so that they are golden brown when passed in the feces. Typical fertile eggs are ovoidal and measure 65 to 75 microns by 35 to 50 microns. Unfertilized female worms produce infertile eggs (fig. 43) that are more elongate than fertile eggs. They measure about 90 by 40 microns and contain an amorphous mass of granules and globules which completely fills the shell. Both fertile and infertile eggs may be found without the outer albuminous coat (fig. 43). These decorticated eggs closely resemble hookworm eggs, but they have a much thicker shell; they contain an undivided cell mass, whereas hookworm eggs usually contain a zygote that is in the four or eight cell stage.

9-16. As the larvae of *A. lumbricoides* migrate through the lungs, pulmonary signs and symptoms appear. These symptoms are most noticeable during the second week after infective eggs are ingested. The most common symptoms of lung migration of larvae are cough, fever, and occasionally blood-tinged sputum. In light infections, the adult worms usually cause little or no problem. In moderate infections there is frequently abdominal pain or discomfort. Large numbers of worms become a burden, and their large size makes them a potential problem. *A. lumbricoides* become very active during febrile diseases and some abdominal conditions. They may attempt to migrate out of the body in both directions, or they may congregate in a tight mass which may block the intestine. The adult worms frequently migrate up the trachea and out of the patient's nose; or they may stimulate the patient to vomit, with the worm being passed in the vomitus.

9-17. When the larvae are migrating through the lungs, you will find many eosinophils and Charcot-Leyden crystals in sputum specimens. You may also find some larvae in the sputum, but you can recover them more readily from gastric washings. After the worms become sexually mature, you can generally find eggs in simple saline

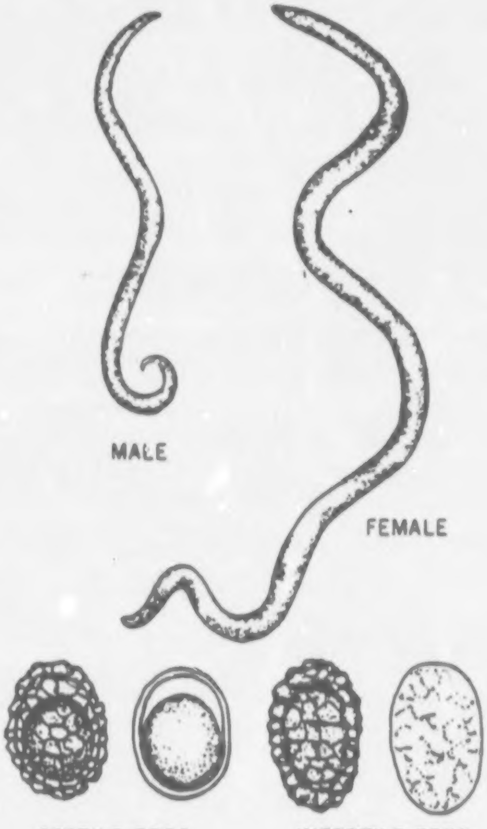


Figure 43. *Ascaris lumbricoides.*

TABLE 6
CHARACTERISTICS OF HOOKWORMS

	A. Duodenale	*N. Americus*
Approximate size, mm.	Male, 10; Female, 12	Male, 8; Female, 10
Position of head	Anterior end continues in same curve as body	Anterior end strongly reflexed dorsally
Buccal capsule	Four conspicuous curved ventral teeth, one pair small teeth deep in capsule	Two ventral semilunar plates, two poorly developed dorsal plates, one median dorsal tooth, and a pair of short triangular lancets deep in capsule
Copulatory bursa	Dorsal ray divided in distal third, each division ends in three digitations (tripartite)	Dorsal ray divided at base, each division ends in two digitations (bipartite)
Copulatory spicules	Two hair-like spicules	Spicules fused at tip into a barb
Vulva	In posterior half	In anterior half

smears of the feces. You do not have to use concentration techniques, because of the large number of eggs produced by each female worm. Detection of single-worm infections presents difficulties because no eggs or the peculiar infertile eggs are found in the stool specimen. You should not rely solely on flotation techniques because infertile eggs are too dense to float with the ordinary techniques. You should always use a direct saline smear in conjunction with any concentration technique.

9-18. **Hookworms.** There are two species of hookworms of major importance to man. *Ancylostoma duodenale* (Old World) and *Necator americanus* (New World). *A. duodenale* is found principally in Southern Europe, Northern Africa, China, and Japan. *Necator americanus* is found in the Southern United States, Central America, the West Indies, and South America east of the Andes, as well as in Central and South Africa, Southern Asia, and Polynesia. The principal factor controlling the distribution of these parasites is temperature. *N. americanus* eggs are quickly killed at temperatures below 45° F., whereas those of *A. duodenale* survive at considerably lower temperatures.

9-19. The life cycles of the hookworms are very similar. The adults attach themselves by their buccal capsules to the mucosa of the small intestine of the host. The females lay a fairly large number of eggs per day; *Necator* produces more than 5,000 and *Ancylostoma* more than 10,000 daily. Passed hookworm eggs are usually in early cleavage and rapidly develop to the first larval stage. When feces containing hookworm eggs are deposited on warm, moist, sandy soil, rhabditiform larvae hatch within 24 to 48 hours. Under favorable conditions, the larvae undergo two molts in about 5 to 8 days. The resulting larvae are third stage filariform larvae which are infective.

9-20. The infective stage filariform larvae of *Necator* infect the human host only by penetrating the skin. The infective larvae of *Ancylostoma* can establish infection after being swallowed or by penetrating the skin. After the larvae penetrate the skin, they enter the blood vessels and are carried to the lungs, where they develop to fourth stage larvae. After about 1 week, they make their way up the pulmonary tree and are swallowed. They attach themselves to the mucosa of the small intestine, where they continue to develop to the adult stage. Then they mate and begin to lay eggs. From the time of skin penetration to egg laying is about 6 weeks. *Ancylostoma* larvae do not undergo any essential development in the lungs, and the filariform infective larvae that are swallowed simply penetrate into the intestinal wall and develop to the fourth stage before they emerge and attach themselves to the intestinal mucosa. Adult worms of both species live and produce eggs for about 5 to 10 years.

9-21. Allergic lesions may develop locally at the site of larval penetration. As with *Ascaris* infections, a single heavy exposure to infective larvae can cause pulmonary symptoms of hacking cough and fever consequent to larval migration through the lungs. This might occur, for example, in troops confined to foxholes or in other situations permitting intimate exposure to large numbers of infective larvae. Iron deficiency anemia is the classical symptom of hookworm disease. It develops only with large worm burdens or in the presence of other conditions that contribute to the depletion of body stores of iron. During World War II, it was found that our troops had relatively light infections and hookworm anemia did not develop. *A. duodenale* generally produces a more severe disease than *N. americanus* when similar numbers of worms are present.

9-22. Diagnosis is made by demonstrating hookworm eggs in stool specimens. You cannot differentiate the species of hookworms on the basis of egg morphology alone. Only on rare occasions will you be required to identify hookworms to species. In the event such identification is required before treatment, set up a "Harada-Mori" culture. After about 1 week check the culture for filariform larvae. Compare the buccal structures under high power. The buccal canal in each is lined with cuticle, but in *Necator* it is thickened at one level to give the appearance of a minute, spear-like structure. The buccal canal of *Ancylostoma* is smooth. With the aid of table 6 and figure 44, you can easily identify the adults as to species. Concentration techniques are not needed to detect the eggs (fig. 44) if infection is heavy enough to produce hookworm disease. On routine fecal examinations, you will recover eggs from patients with only one or two egg-producing worms with either the zinc-sulfate centrifugal flotation concentration method, the formalin-acid-ether concentration method, or the Kato thick smear method.

9-23. To give the physician some idea of the significance of the infection, you should perform a simple egg count. Make a saline direct smear using sufficient feces (2 mg.) to cover the tip of an applicator stick. Systematically cover the entire smear and count all of the eggs. Fewer than five eggs per smear indicates light infection that ordinarily does not produce anemia. Counts of 20 or

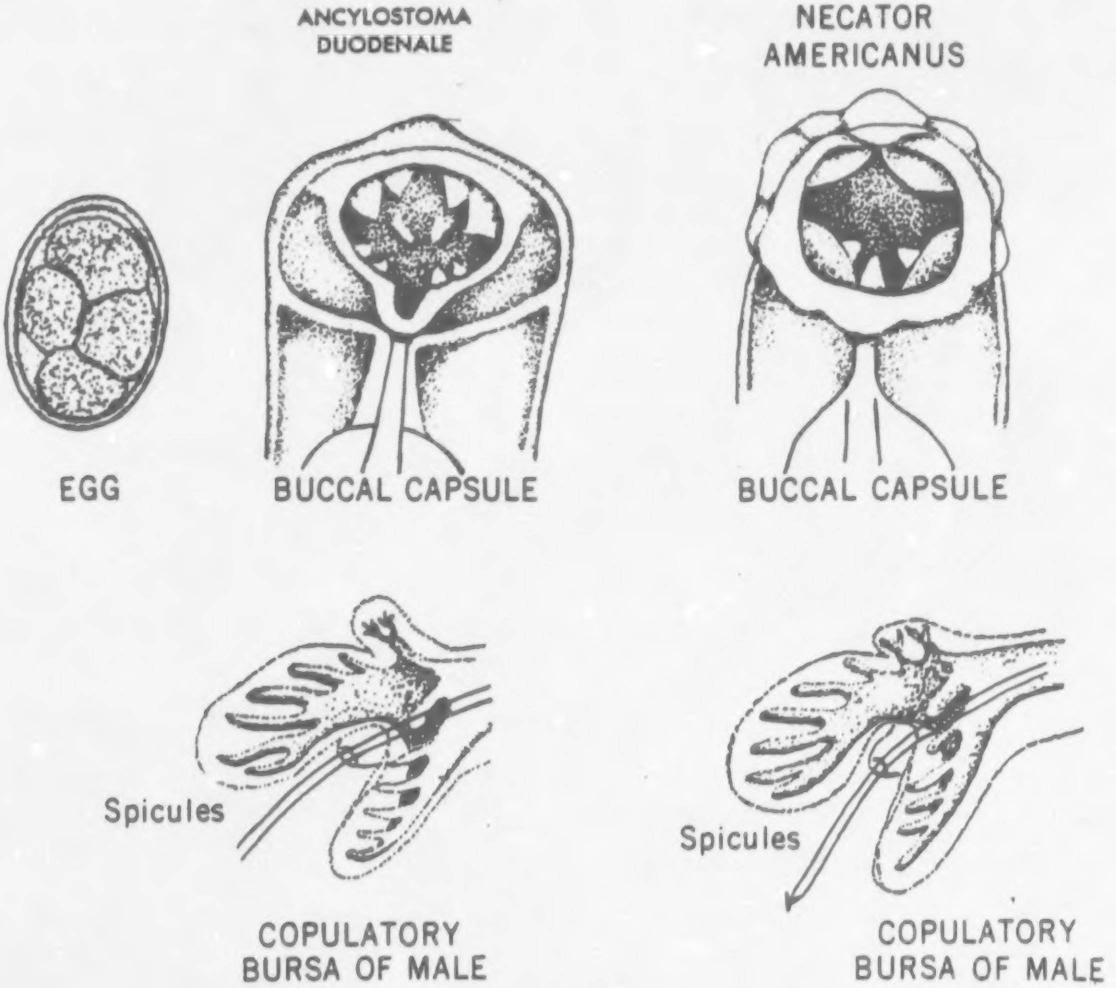


Figure 44. *Hookworms.*

more eggs per smear indicate clinical significance. Very heavy infections will produce over 100 eggs per smear. Such heavy infections usually cause an observable anemia.

9-24. **Strongyloides Stercoralis.** *Strongyloides*, like the hookworm, requires warm, moist soil for completion of its life cycle. In general, the distribution of *Strongyloides* parallels that of human hookworms. Its distribution pattern is much more spotty than that of the hookworms and fewer people are infected. It is found mainly in tropical and subtropical areas. Cases are rarely reported from Europe. In the United States, *S. stercoralis* is endemic along the Gulf Coast of Florida, Louisiana, Mississippi, and Alabama.

9-25. *Strongyloide stercoralis* has a rather complicated life cycle. The parasitic adults live in the mucosa of the small intestine. Eggs are passed directly into the mucosa, where they embryonate and hatch. Rhabditiform larvae reach the lumen of the intestine and are passed in the feces. Once they are passed, they may follow either a direct or indirect route of development. The indirect route of development occurs when larvae develop into free-living adult male and female worms. This usually happens when the rhabditoid larvae are deposited on warm, moist, shaded soil. The free-living adult females produce eggs, and 2 to 3 days later free-living rhabditoid larvae hatch. Within 24 hours after the eggs hatch, the rhabditoid larvae develop into infective filariform larvae. Species of *Strongyloides* which parasitize man have only one completely free-living generation, but some species that parasitize other mammals may have more than one free-living generation. The direct mode of development usually occurs when drier and cooler conditions are prevalent. Under such conditions, the free-living rhabditoid larvae that are deposited on the soil develop directly to infective filariform larvae, thus bypassing the free-living adult stage. The filariform larvae, regardless of mode of development, may live for as much as 2 weeks on the soil.

9-26. On contact, the infective filariform larvae penetrate the skin and enter the small blood vessels, through which they are carried to the lungs. In the lungs they break out of the capillaries into the air spaces and then make their way to the intestine. They enter the mucosa of the small intestine, molt twice, and mature in about 2 weeks. There are no males in the parasitic stage; there are only females. The adult female worm is parthenogenetic (produces viable eggs without fertilization by a male worm). In some cases the rhabditoid larvae transform into infective filariform larvae before they are passed from the intestine of the host. When that happens, the infective larvae penetrate the intestinal wall (autoinfection); and hyperinfection of the host occurs.

9-27. The tunneling process in the small intestine and the tissue reaction to eggs, worms, and larvae cause epigastric pain, abdominal cramps, and diarrhea. Symptoms may resemble those of peptic ulcer. Significant eosinophilia is present in about half of the cases. Patients with hyperinfection may have fever and other generalized symptoms. Severe infections, especially if associated with other debilitating conditions, can lead to death.

9-28. Diagnosis is usually made by finding the motile rhabditoid larvae (fig. 45) in feces or duodenal contents. The larvae are large (0.25 mm. to 0.40 mm.) and actively motile. You can see them easily on direct saline smears with the aid of a microscope. In suspected cases where you do not find the larvae on the direct smear, set up a Baermann apparatus (fig. 46). Cover a piece of filter paper with feces. Place the filter paper, feces side down, on the gauze. Fill the funnel to the level of the filter paper with water. Incubate at 37° C. to 40° C. for 1 hour. The larvae will migrate from the feces into the water and settle to the bottom.

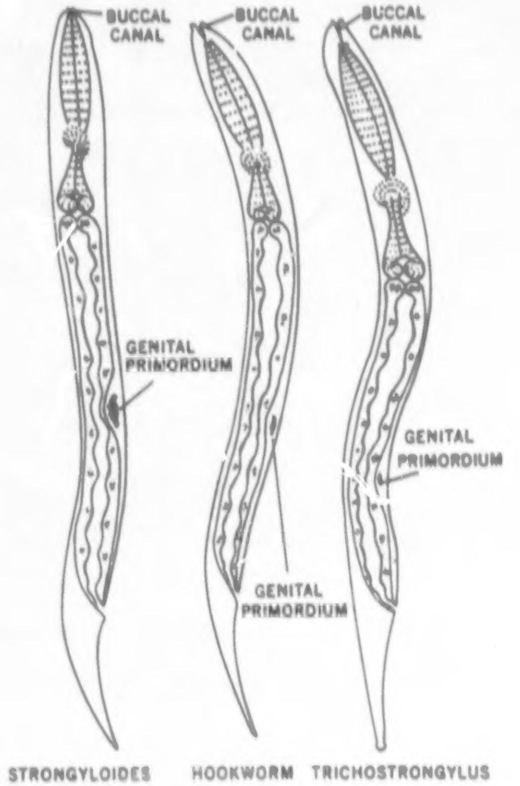


Figure 45. Rhabditoid Larvae.

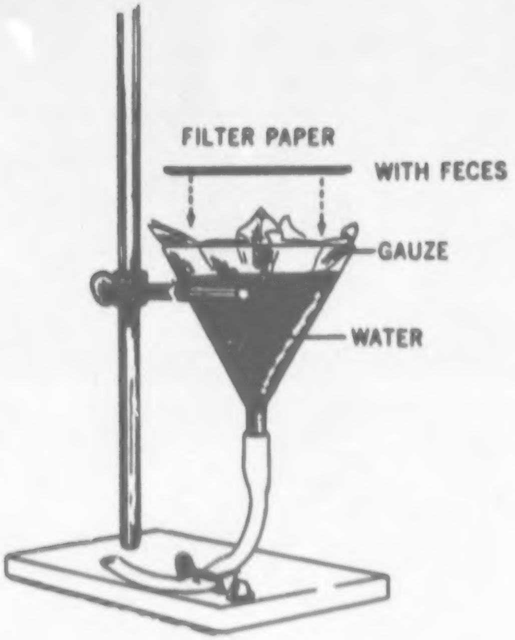


Figure 46. Baermann apparatus.

Draw off 10 cc. of water from the bottom of the funnel and centrifuge. Examine the sediment for typical larvae. Examine fecal specimens as soon after passage as possible. If hookworm eggs are present, motile larvae will hatch within 24 hours when the stool is allowed to stand at room temperature. Also, the rhabditoid larvae of *Strongyloides* will develop into filariform larvae in that period of time. On rare occasions, larvae may be found in sputum, urine, or aspirates from body cavities.

9-29. The points to be especially noted to differentiate the rhabditoid larvae of *Strongyloides* from those of the hookworms (fig. 45) are:

- The buccal canal of *Strongyloides* is very short, whereas that of the hookworms is longer and narrow.
- The genital primordium of *Strongyloides* is much larger than that found in the hookworms.

When examining filariform larvae (fig. 47), note the long, slender body shape and the relatively long esophagus, which is approximately half the body length. The filariform larva of *Strongyloides* can be distinguished from a filariform larva of hookworm because *Strongyloides* has a notched tail, whereas a filariform larva of hookworm has a pointed tail.

9-30. **Trichinella Spiralis.** *Trichinella* has a worldwide distribution. The principal reservoir for human infection is the pig. As expected, the parasite is most commonly found in pork-eating populations. *Trichinella* has traditionally been a serious problem in parts of Europe and the United States until recent decades. It is still widely distributed in Germany, Poland, Spain, Hungary, and the lower Danube countries. There are still reports of small epidemic outbreaks occurring in the United States and Latin America. In the last few years there have been several outbreaks reported from Thailand.

9-31. *Trichinella* is unique among the intestinal nematodes that parasitize man. Its life cycle does not include any developmental stages outside the body of a host, and it does not involve a true intermediate host. It develops about equally well in man, pigs, rats, and many other mammals. Man usually gets his *Trichinella* infections by eating uncooked portions of an infected pig, and the pig could just as readily acquire infection by eating uncooked portions of an infected human.

9-32. The cycle in man begins when he ingests meat, usually pork, that contains infective larvae. In the small intestine, the cyst that surrounds the larvae is digested; and the larvae enter the intes-

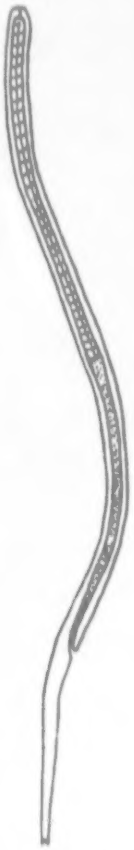


Figure 47. Filariform larvae of *Strongyloides*.

tinal crypts. The larvae mature very rapidly. The adults (fig. 48) are small white worms, just visible to the unaided eye, the male being 1.5 mm. long and the female about 3.9 mm. long. The male dies after mating and is passed from the intestine in a very short time. Upon fertilization, the female burrows more deeply into the intestinal mucosa and by the 5th to 7th day begins to deposit larvae directly into the mucosa. A single female gives birth to several hundred larvae over a period of 4 to 16 weeks or more. The little larvae measure 100 microns long and 6 microns in diameter. They reach the mesenteric venules and lymphatics and become distributed to all parts of the body. The young larvae leave the capillaries and invade voluntary (striated) muscle. This is the only tissue in which the larvae are able to develop and grow. The muscles most frequently affected are in the diaphragm, larynx and tongue, and the biceps, gastroenemius, and deltoid muscles. One to two weeks after exposure, most of the larvae have reached striated muscle. About 3 weeks after exposure, the larvae in the muscles (fig. 48) have grown to about 1 mm. in length. At this stage they have become coiled and encapsulation has begun. The larvae are infective for another host when they reach the coiled stage. Calcification of the capsule begins after about 8 weeks. In 9 to 12 months most of the encapsulated larvae are completely clacified and dead, but some may live for several years.

9-33. The severity of the disease caused by *Trichinella spiralis* is related to the number of larvae ingested. Ingestion of a small dose of larvae probably goes unrecognized. In heavier infections, as the young worms excyst and migrate into the intestinal mucosa, the patient exhibits symptoms of gastroenteritis. As the worms mature and release young larvae, acute inflammatory reactions occur around the larvae as they become temporarily trapped in capillaries of various organs during their migration through the tissues. An eosinophilia of 15 to 50 percent develops during this period. Patients with severe infections may experience symptoms affecting special muscle groups, such as painful swallowing, breathing, or chewing. In very heavy infections, as the larvae continue to be produced and continue to invade the muscles, the outcome may be fatal; or permanent crippling of the patient may result.

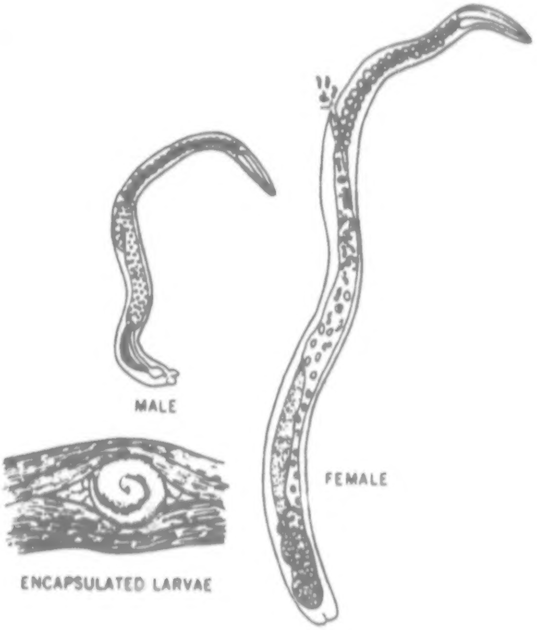


Figure 48. *Trichinella spiralis.*

9-34. A definitive diagnosis of *Trichinella* is very difficult to make in the early stages of the disease. During the very early stages, the physician has nothing to go on but the patient history. At that point the patient will have an eosinophilic leukocytosis. You can sometimes find *T. spiralis* larvae in the centrifuged sediment of hemolyzed blood during early phases of heavy infections or in the cerebrospinal fluid when CNS involvement is present. Larvae can usually be found in suspected meat if it is still available. Compress a portion of the tissue between two glass slides before examining it with the microscope, and you will be able to easily see the encapsulated larvae. If large amounts of tissue are available, digest it with gastric juices and then examine the sediment for freed *Trichinella* larvae. As the infection advances and larvae reach the muscles (7 to 14 days), it is possible to recover them from muscle biopsies. Use the compression slide technique for muscle biopsies as well as examining suspected meat. Skin test antigens are available for *Trichinella*, and the physician will occasionally use them as an aid in diagnosing the disease. Reliable agglutination procedures are available for this infection. A bentonite flocculation test and an indirect hemagglutination test have been found to be sensitive and satisfactorily specific. The tests, when performed on acute and convalescent serum, will show a significant increase in titer.

10. Filariae and Dracunculus

10-1. Filarial worms live as adults in the blood and lymphatic circulatory systems, muscles, connective tissues, and body cavities of vertebrates. *Dracunculus medinensis* is similar to the filarial worms, but they are not taxonomically related. However, it is convenient to consider it in this section.

10-2. **Filariae.** The true filarial worms are unique because they have an embryonic stage

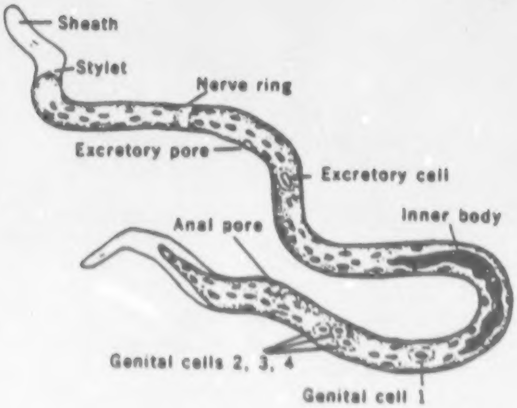


Figure 49. Microfilaria.

known as a microfilaria (fig. 49), which is deposited directly into the tissues by the females. The young embryos migrate through the tissues to the blood or lymphatic vessels, and they periodically appear in the vessels of the skin. In some species, the microfilariae retain the shell of the egg as a sheath, and in other species they actually hatch from the egg. Bloodsucking insects, while taking a blood meal, take in some microfilariae. In appropriate bloodfeeding insects, microfilariae undergo a period of development and become infective third stage filariform larvae. When the infected insect takes another blood meal, the larvae escape from the proboscis and enter the skin through the bite wound. The larvae then require several months of development to become mature worms.

10-3. *Wuchereria bancrofti. W. bancrofti* is widely distributed in most tropical areas of the world. In some of the South Pacific islands and parts of India the parasite is endemic; however, in most of the areas where the worm is widespread, relatively few individuals are infected. Man is the only known host.

10-4. *W. bancrofti* is a small, thread-like worm (fig. 50). The males measure about 4 centimeters long and the females about 8 centimeters long. The adults usually live in lymph nodes and lymphatic vessels in the groin and external genitalia. The microfilariae commonly circulate in peripheral blood only at night *(nocturnal* periodicity). Strains of the parasite from the South Pacific islands demonstrate practically no periodicity. The parasite is transmitted by many species of culicine and anopheline mosquitoes.

10-5. In the early stages of infection there are periods of fever, pain, and some swelling of the lymph nodes and lymph channels. In chronic cases, the lymph vessels become obstructed; and extensive growth of connective tissue develops in the groin, external genitalia, and legs, producing disfigurement (elephantiasis). Only a small percentage of the acute cases continue to the chronic stage of elephantiasis.

10-6. The demonstration of microfilariae or adult worms is the only proof of filariasis. Techniques for demonstrating microfilariae of *W. bancrofti* in blood specimens also apply to other filariae whose microfilariae circulate in the blood. A careful search for microfilariae should always be made when filariasis is suspected, and examinations should be repeated at intervals. Microfilariae of *W. bancrofti* may be found in preparations of blood or aspirated contents of lymph nodes or hydroceles or in chylous urine. In areas where *W. bancrofti* demonstrates nocturnal periodicity, microfilariae are most readily detected in blood taken at night. In the South Pacific islands, the nonperiodic microfilariae may be slightly more numerous in the daytime than at night.

10-7. In well-established infections, microfilariae may be seen microscopically, thrashing about in a drop of fresh blood. You should mix one drop of blood with one or two drops of physiological saline on a glass slide; spread the preparation and cover it with a 22 x 50 mm. coverslip. Use the low power objective for scanning the slide. Wet preparations are good to use for screening purposes. When you find microfilariae, prepare thick or thin stained blood smears to identify the species. It may be necessary, particularly in new infections, to use one of the concentration methods to detect microfilariae. The Knott technique is most commonly employed. The procedure is very simple and effective. Withdraw 2 cc. of blood from a vein and dilute it in 10 cc. of 2 percent formalin. Mix in a 15 cc. centrifuge tube, centrifuge for 5 minutes at 2,000 rpm, decant the supernant, and examine the sediment for microfilariae. You can spread, dry, fix, and stain the sediment with Giemsa as you did the thick or thin blood smears. Table 7 and figure 51 give the information necessary to identify the species in stained films. The

MALE
FEMALE

Figure 50. *Wuchereria bancrofti.*

TABLE 7
CHARACTERISTICS OF MICROFILARIAE

SPECIES	FOUND IN	LENGTH (IN MICRONS)	PERIODICITY	SHEATH	TAIL MORPHOLOGY
W. bancrofti	Blood	245-295	Usually Nocturnal	Present	Tapers to delicate point; Nuclei do not extend to tip of tail.
B. malayi	Blood	177-230	Nocturnal	Present	Tapers to delicate point; Two terminal nuclei.
L. loa	Blood	250-300	Diurnal	Present	Tapers gradually; Nuclei continuous into tail.
M. ozzardi	Blood	185-200	None	Absent	Tapers gradually; Nuclei do not extend to tip of tail.
D. perstans	Blood	190-200	None	Absent	Tapers gradually; Bluntly rounded; nuclei to tip of tail.
D. streptocerca	Skin	180-240	None	Absent	Slender hooked tail; Bluntly rounded; nuclei to tip of tail.
O. volvulus	Skin	150-287 or 285-368	None	Absent	Tapers gradually; Nuclei do not extend to tip of tail.

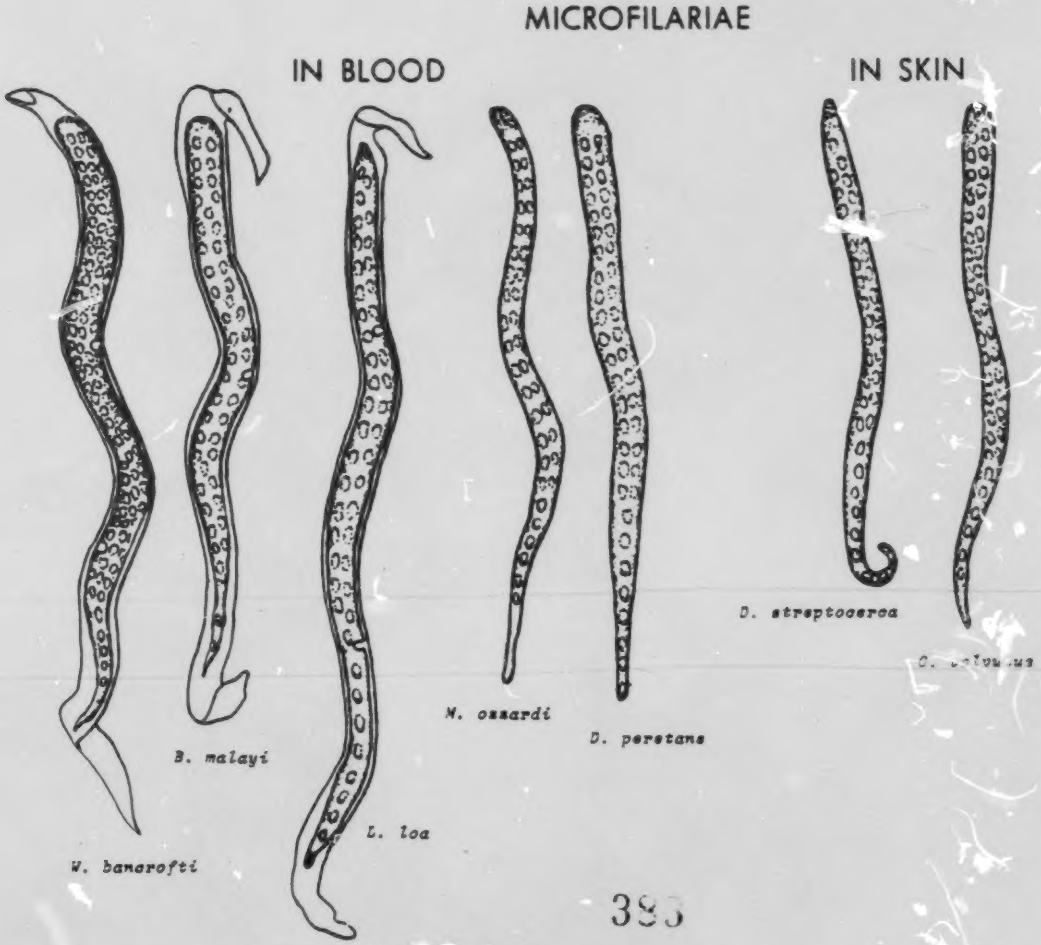

MICROFILARIAE
IN BLOOD
IN SKIN
W. bancrofti
B. malayi
L. loa
M. ozzardi
D. perstans
D. streptocerca
O. volvulus

Figure 51. Microfilariae.

demonstration of adult worms in lymph node biopsies gives verification of the clinical diagnosis even in the absence of microfilariae. Biopsies are not recommended as a routine diagnostic procedure. In the event that biopsies are taken, they should be studied by a trained pathologist and parasitologist.

10-8. Other laboratory studies are sometimes helpful in filariasis. Leukocytosis and eosinophilia may be present, but they are not specific. Immunological tests of all kinds have been employed, but skin tests and complement-fixation tests have received the most attention. The antigens usually employed are derived from *Dirofilaria immitis*, a filariid which occurs in dogs. The tests are not species specific, and the techniques have not been standardized; however, results of these tests may be helpful in conjunction with other findings. They are especially useful when testing large groups of patients as in surveys. They require very critical evaluation in individual cases.

10-9. ***Brugia malayi.*** *B. malayi* is found in Southeast Asia. Its range extends from Ceylon northward into India and from Indonesia and Borneo through Malaya and Thailand to South Korea. *Brugia malayi* occurs naturally in cats and monkeys, as well as man. The adult parasites live in lymph nodes and vessels and produce the same type of symptoms and disease as *Wuchereria bancrofti*. *B. malayi* adults and microfilariae are very similar to *Wuchereria bancrofti*, but they differ sufficiently to be assigned to two different genera. Adult *B. malayi* are about half as large as *W. bancrofti*. The microfilariae of both species (table 7 and fig. 51) are sheathed. *B. malayi* microfilariae have two cells in the tip of the tail which are not present in *W. bancrofti*. The microfilariae of *B. malayi* usually exhibit nocturnal periodicity just as those of *W. bancrofti* do, but the periodicity does not appear to be as strong in some areas.

10-10. ***Loa loa.*** *Loa loa* is commonly known as the eye worm or the missionary worm. It is found in central and western Africa. The principal endemic areas lie on the coastal plains from Sierra Leone to Angola and along the watersheds of main rivers. They are medium sized worms (males 30 to 35 mm. long and females 50 to 70 mm. long) that inhabit subcutaneous tissues of humans. As the worms migrate around in the subcutaneous tissues, "calabar" swellings appear and remain for 2 to 3 days. The painful swellings are believed to be allergic responses to the worms. On occasions, adult worms migrate beneath the conjunctiva of the eye. The parasite causes no serious damage to the host, but it is rather disturbing for a worm to migrate across the eye.

10-11. Flies of the genus *Chrysops* are the intermediate hosts of *Loa loa*. You probably know them as "tabanid" or "deer" flies. They usually bite man between dawn and dusk—the same time that the microfilariae of *Loa loa* are found in the peripheral circulation. The appearance of microfilariae in the peripheral circulation only during the daytime is called *diurnal* periodicity. The microfilariae (table 7 and fig. 51) are sheathed and have a core of nucleated cells that extends without interruption into the tip of the tail. When you are unable to find microfilariae in suspected cases, the patient should be skin tested with a filarial antigen. An immediate positive reaction will almost always occur in people who have the parasite.

10-12. *Onchocerca volvulus. O. volvulus* has a very irregular distribution in the African and American tropics. It is not endemic in coastal areas. The principal endemic foci in Africa are inland, along river courses, whereas in Central and South America the disease is confined to coffee plantations at elevations of 1,000 to 3,500 feet. The adult worms are quite long and slender (males 19 to 42 mm. long by 130 to 210 microns, females 335 to 500 mm. long by 270 to 400 microns). They usually live in pairs in subcutaneous nodules. The nodules may occur on any part of the body. In Africa the nodules frequently occur over any bony prominences of the body, whereas in Central and South America they are found more frequently on the head or scalp. The microfilariae are not sheathed. The microfilariae of *Onchocerca* remain in the superficial lymphatic spaces and connective tissue of the skin after they escape from the nodules. They rarely, if ever, get into the bloodstream.

10-13. The insect intermediate hosts belong to the genus *Simulium*, commonly known as black flies. Members of the genus *Simulium* are blood suckers, but they have chewing mouthparts. To take a blood meal, they chew a hole in the skin to the capillary bed. They lap up the blood, and at the same time, the tissue juices which contain the microfilariae of *O. volvulus*.

10-14. The nodules of *Onchocerca volvulus* cause little or no inconvenience. As the microfilariae migrate about through the skin, sensitization reactions occur. In addition to itching, the skin becomes dry, wrinkled, rough, and shining. Eventually the skin thickens and loses its elasticity. In some areas there is a very high incidence of blindness among people who have *Onchocerca volvulus* infections. Blindness is a result of the damage caused by the microfilariae migrating through the eye.

10-15. The microfilariae (table 7 and fig. 51) do not enter the bloodstream; therefore, you cannot find them by examining blood smears, not even with concentration methods. Since the microfilariae are in the skin, the examination of skin snips is the most practical diagnostic measure. Remove a thin skin shaving about 0.5 cm. in diameter with a razor blade, tease the specimen apart in a drop of saline, and examine it microscopically for microfilariae. Excised nodules should be examined for microfilariae and/or adult worms.

10-16. *Dipetalonema (=Acanthocheilonema) perstans. Dipetalonema perstans* is quite common in the tropical regions of western and central tropical Africa and northern South America. It apparently is a harmless parasite that lives in body cavities. Biting gnats belonging to the genus *Culicoides* serve as intermediate hosts. The microfilariae (table 7 and fig. 51) circulate in the peripheral blood. You will have to differentiate them from the microfilariae of the more harmful species.

10-17. *Dipetalonema (=Acanthocheilonema) streptocerca. Dipetalonema streptocerca* is common in the same areas as *Onchocerca volvulus.* Species of *Culicoides* serve as intermediate hosts for this parasite. Adult worms live in cutaneous connective tissue, and the microfilariae found in the skin are similar to the microfilariae of *O. volvulus.* The microfilariae (table 7 and fig. 51) of *D. streptocerca* are most frequently found in skin snips, but you must make the snip deep enough to include dermal tissue. Most infected persons are symptomless, but they may have some cutaneous edema and disfigurement.

10-18. *Mansonella ozzardi. M. ozzardi* occurs only in Central America, South America, and the West Indies. Species of the genus *Culicoides* and possibly species of the genus *Simulium* serve as intermediate hosts. The adult worms live in body cavities and apparently cause no harm. The microfilariae (table 7 and fig. 51) are recovered from peripheral blood.

10-19. **Dracunculus Medinensis.** *D. medinensis,* known as the guinea worm or fiery serpent, is found in man throughout tropical Africa and especially in much of the Middle East. It has been reported from horses, cattle, leopards, polecats, and monkeys from the endemic areas. It is found in furbearing animals in North America and dogs in China.

10-20. *Dracunculus* is similar to the filarial worms, but there are fundamental differences. The adult female *Dracunculus* is much larger than the filarial worms. *Dracunculus* females pass first stage rhabditoid larvae rather than pre-larval embryos (microfilariae). The larvae are passed from the host and do not get into the skin or into the circulatory system of the host.

10-21. The adult female *Dracunculus* lives in the subcutaneous tissues, usually on the arms or legs of man. When ready to release larvae, the female causes a blister to form in the skin of the host. A loop of the uterus of the worm lies very near the blister and when the blister comes in contact with water in a stream, lake, pond, etc., the blister bursts. The uterus then prolapses and ruptures, releasing large numbers of larvae into the water. The larvae must be ingested by copepods belonging to the genus *Cyclops,* which serve as intermediate hosts. In the copepod the larvae develop to infective third stage larvae in about 3 weeks.

10-22. People become infected with *Dracunculus* by drinking water that contains the minute infected *Cyclops.* When the infected *Cyclops* reaches the intestine, the larvae are freed as the copepod is digested. The larvae make their way through the intestinal wall and into the body cavity. There they develop to maturity in 8 to 12 months. The cycle is completed when the female becomes gravid and migrates to the subcutaneous tissues.

10-23. As the adult female migrates to the subcutaneous tissues, there may be pronounced allergic reactions such as skin rashes, nausea, vomiting, and diarrhea. After the female worm reaches the skin and the blister ruptures, there is a good chance for secondary bacterial infection in the worm channel.

10-24. A positive diagnosis cannot be made until the worm forms a blister on the skin. At times, you may demonstrate the larvae by placing the blister in contact with water and allowing it to rupture. Of course, you can make a specific diagnosis as soon as you find the worm protruding from the subcutaneous channel.

11. Zoonotic Nematodes

11-1. Parasite infections that are maintained at more or less stable rates in wild and domestic animal populations are said to be enzootic. When a human is infected with an animal parasite, it is known as a zoonotic infection. They are only incidental parasites of humans. Some have limited geographical distributions, whereas others are quite widespread. The possibility that military operations may extend into areas where personnel incur a greater risk of exposure to zoonotic infections is ever present. Furthermore, the military medical service is often called upon to provide medical care for native populations. Your knowledge of these parasites and the various tools of diagnosis will be a great help to physicians who must diagnose and treat patients with zoonotic infections.

11-2. In 1969, Beaver* established four categories of relationships that exist among various zoonotic helminth infections:

---

* Beaver, P. C. 1969. *The Nature of Visceral Larva Migrans.* J. Parasit. 55:3–12.

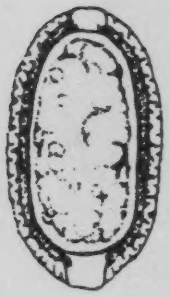


Figure 52. Egg of *Capillaria hepatica.*

(1) Category I: Humans are normal and adequate, though unnatural, *final* hosts.
(2) Category II: Humans are more or less normal, but inadequate, *final* hosts.
(3) Category III: Humans are more or less normal, but unnatural, *paratenic* hosts.
(4) Category IV: Humans are more or less normal, but unnatural, *intermediate* hosts.

The first three of the categories are used here to organize the zoonotic nematode infections which make up this section. None of the nematodes discussed in this section fits Category IV, which applies mainly to larval tapeworms.

11-3. **Category I.** The first category includes parasites for which humans are normal and adequate, though unnatural, final hosts. The nematodes of this group develop and behave in man essentially as they do in their natural final hosts. Some of the nematodes in this group are very common and are of considerable importance in certain localities. *Capillaria philippinensis* will be discussed in this section, even though at the present time it is not known if it is a zoonotic infection. It was only recently reported, and very little is known of its life history.

11-4. *Capillaria philippinensis. C. philippinensis* is the only intestinal capillariid known to parasitize man. As the name indicates, this parasite is found in the Philippines. Several thousand cases have been reported since the parasite was first discovered in 1964. All cases reported to date have been from the Philippines.

11-5. The small adult worms (less than 5 mm. long) are found imbedded in the mucosa near the junction of the large and small intestines. The eggs are very similar to those of *Trichuris trichiura;* therefore, it will be necessary to measure the eggs to differentiate the two parasites. The eggs of *T. trichiura* measure 50 to 54 by 22.5 microns, whereas those of *C. philippinensis* measure about 36 to 45 by 21 microns.

11-6. Thus far, none of the details of the life cycle of *C. philippinensis* have been reported. A large percentage of the people infected with this parasite have very severe symptoms, and many deaths have been attributed to infection with *C. philippinensis.* Many of the patients have been weak and very emaciated when first seen for treatment. Intractable diarrhea has been a common symptom.

11-7. *Capillaria hepatica. C. hepatica* is a delicate thread-like parasite that lives in the parenchyma of the liver of mammals, especially rodents. The female worms pass eggs directly into the liver tissues, causing fibrosis. As the worms and eggs accumulate in the liver, considerable damage results. To make a positive diagnosis, a liver biopsy must be performed to recover the worm or the typical eggs. Morphologically the eggs (fig. 52) are similar to those of *T. trichiura.* They measure 51 to 61 by 30 to 35 microns, which is slightly larger than those of *T. trichiura.* The eggs of *C. hepatica* are more barrel shaped, and the shells appear velvety, because they are finely pitted with minute pores. You may occasionally find these eggs in the feces of human patients. Do not confuse them with *T. trichiura.* The presence of the eggs in feces does not mean that the patient has *C. hepatica,* it only means that the patient has recently eaten infected liver. The eggs of *C. hepatica* are not infective until they have undergone a period of embryonation in damp, shaded soil. Mammals become infected when they ingest *infective* eggs.

11-8. *Ancylostoma ceylanicum. A. ceylanicum* is a hookworm that normally parasitizes dogs and cats in Southeast Asia and Brazil. In some areas, it is a more common parasite of humans than the other hookworms. The adult parasites are about 30 percent smaller than *A. duodenale,* and the buccal cavity and bursa are characteristic for the species. The larval stages are very similar to those of *A. duodenale,* and the eggs are indistinguishable from the other hookworm eggs.

11-9. *Ternidens deminutus.* The natives of Southern Rhodesia, Malawi, and Mozambique are commonly infected with *T. deminutus.* The parasite resembles a hookworm; but the buccal capsule is terminally located, and the buccal cavity is guarded on the inside by a double row of stiff bristles. The worms attach themselves by inserting their heads into the intestinal wall. Eggs are passed in feces and hatch in the soil. After a period of development, the larvae become infective. Infection occurs when infective, third stage larvae are ingested. The eggs of *Ternidens* can be distinguished from hookworm eggs only because they are larger. *Ternidens* eggs average 84 by 51 microns, whereas hookworm eggs may be as large as 70 by 40 microns. You should not confuse *Ternidens* with hookworms, because the usual forms of hookworm treatment are only moderately effective against *Ternidens*.

11-10. *Syngamus*. Members of the genus *Syngamus* are small nematodes that live in the respiratory tract of birds and mammals. The males and females are permanently joined together for mating. Man is an incidental host for *S. laryngeus* and possibly *S. nasicola*. Human infections have been reported from Puerto Rico, Brazil, the West Indies, and the Philippines. The adult worms irritate the upper respiratory tract, causing coughing, sneezing, and an asthma-like condition. Diagnosis is made by finding the adult worms or eggs in sputum or feces. The eggs are ovoidal, and the shell appears to be made up of a large number of prisms cemented together. Be aware of these parasites, and do not confuse the eggs with pollen grains that are commonly observed in sputum and feces.

11-11. *Trichostrongylus*. Members of the genus *Trichostrongylus* are typically found in ruminants, attached to the small intestine. They are considered incidental parasites of man; however, *T. orientalis* is more commonly found in man than in other animals in Japan and Korea. Trichostrongyles are found in man in many areas of the world, including Africa, Iran, Iraq, India, Armenia, Siberia, Indonesia, Japan, Korea, China, Formosa, Australia, and (rarely) the United States.

11-12. Eggs are passed in feces. They require moisture, warm temperature, shade, and grass or carpet vegetation for proper development. Rhabditoid larvae hatch in the grass, and after a period of development they become infective. The larvae cannot infect a host by penetrating the skin; they must be ingested. Contaminated green vegetables are the main source of infection. The larvae burrow into the intestinal wall, and a few days later they re-emerge without making a lung migration. They attach themselves by inserting the anterior part of their body into the intestinal wall, where they remain to develop to maturity. Infections usually consist of only a few worms, but heavy infections can occur. The usual infections cause no symptoms, but heavy infections cause symptoms similar to hookworm disease.

11-13. Diagnosis is based on finding characteristic eggs in stool specimens. These eggs are also very similar to those of hookworms. They are larger than hookworm eggs, and they are more pointed at one end. To confirm the diagnosis, it is necessary to measure the eggs. *Trichostrongylus* eggs measure 70 to 90 by 40 to 50 microns, whereas hookworm eggs measure up to 70 by 40 microns. You can cultivate eggs and identify the larvae. The first and second stage larvae are rhabditoid. They are similar to the rhabditoid larvae of hookworms, but they can be readily distinguished because they have a minute bead-like knob at the tip of the tail (fig. 45). You should make every effort to properly identify the parasite because *Trichostrongylus* does not respond to the usual hookworm treatments.

11-14. *Thelazia*. Two species of the genus *Thelazia* have been reported from the conjunctival sac of man. *Thelazia callipaeda* has been reported from several oriental countries. *Thelazia californiensis* has been reported from the western states of the United States. These parasites normally inhabit the conjunctival sac of dogs and other mammals. They are only incidental parasites of man. The worms measure about 4.5 mm. by 0.25 to 0.85 mm. Females produce clear, thin shelled, embryonated eggs which measure 57 by 35 microns. Certain species of flies serve as intermediate hosts. The worms have a rough cuticle which causes considerable damage to the tissues of the eye as they crawl back and forth across the front of the eye. Diagnosis depends upon the identification of the worm after it is removed from the anesthetized eye.

11-15. *Physaloptera*. *Physaloptera caucasica* is the only species of the genus *Physaloptera* that is known to parasitize man. It is a natural parasite of the intestinal tract of some monkeys. It has been reported from man in the Caucasus, tropical Africa, Southern Rhodesia, India, Panama, and Columbia. The adults are found attached to the walls of the esophagus, stomach, and small intestine. If you do not examine them carefully, you may confuse them with *Ascaris*. The eggs have thick, smooth shells; and they are fully embryonated when passed in the feces. They measure 44 to 65 microns by 32 to 45 microns. Infertile *Physaloptera* eggs resemble fertile decorticated *Ascaris* eggs; however, fertile *Physaloptera* eggs contain a fully developed larva, whereas the eggs of *Ascaris* never contain a larva when passed in feces. Examine several eggs carefully to properly differentiate the two.

11-16. *Dioctophyma renale*. *Dioctophyma*, commonly known as the giant kidney worm, is widely distributed throughout the world. It is primarily a parasite of fish-eating carnivores, but it is occasionally found in horses, cattle, and humans. *Dioctophyma* adults are very large, reddish nematodes which inhabit and destroy the parenchyma of the kidney. Males measure from 150 to 400 mm. long, and females measure up to 1,000 mm. long. Females pass eggs that are unembryonated, dirty brown in color, have deep pittings in the shell except at the poles, and measure 64 to 68 microns by 40 to 44 microns. Diagnosis is made by finding typical eggs in *urine*.

11-17. *Gongylonema pulchrum. G. pulchrum* inhabits the mucosa of the esophagus of ruminants. It has also been found in pigs, bears, hedgehogs, monkeys, and occasionally in man. The parasite has been reported from most areas of the world. In man the worm has been found in the mucosa and subdermal connective tissues in the vicinity of the mouth, not the esophagus. It produces local irritation and inflammation. The adult worms are thread-like, and the females are larger than the males. They may measure up to 145 mm. long. The females lay fully embryonated eggs which are transparent, thickshelled, broadly ovoidal and measure 50 to 70 microns by 25 to 37 microns. Varous species of dung beetles and cockroaches serve as intermediate hosts. The definitive host is infected by swallowing an infected insect.

11-18. *Acanthocephala.* Acanthocephala is the phylum commonly known as thorny-headed worms. They are not nematodes, but they are included here for the sake of convenience. They are parasites of the intestinal tract of vertebrates. They firmly attach themselves to the intestinal wall by a retractable proboscis that is covered with hooks. The worms are not segmented; they have no digestive tract and the sexes are separate. Species of this phylum require an intermediate host to complete their life cycles. *Macracanthorhynchus hirudinaceus*, normally a parasite of pigs, and *Moniliformis moniliformis*, normally a parasite of rodents, are the only two species of the phylum that have been reported from man. Reports of these parasites in man have been very rare.

11-19. **Category II.** In the second category, humans are more or less normal, but inadequate, final hosts. Some nematodes that usually infect other animals can infect humans, but they do not complete their normal development. The parasites behave and develop in humans just as they do in natural final hosts until certain stages are reached. Most of these parasites are stymied in their development before they begin to deposit reproductive products. They usually lose their way and end up in abnormal sites in humans. These factors make it very difficult to arrive at a proper diagnosis.

11-20. *Dirofilaria. D. immitis* is the heartworm of dogs. It is a common parasite of dogs throughout most of the tropical and subtropical areas of the world. *D. immitis* utilizes mosquitoes as intermediate hosts. The parasite has occasionally been found encapsulated in the lung of humans. They have been found on radiographic examinations as coin lesions which required surgery to rule out a malignancy. Many of the worms have been found to be sexually mature, but no microfilariae of *D. immitis* has been found in the blood of humans. Skin tests and complement-fixation tests employing *Dirofilaria* antigens may be useful in cases where typical coin lesions are discovered.

11-21. There is another group of *Dirofilaria* that are found in tumors and abscesses in the subcutaneous tissues and tissues of the eyes of humans. They have been reported from most areas of the world. The worms are collectively referred to as *Dirofilaria "conjunctivae."* As with *D. immitis* in humans, *D. "conjunctivae"* often reaches sexual maturity; however, they rarely produce microfilariae, and the microfilariae are not found in the blood of humans. The various species of *Dirofilaria* that cause these infections in man are normally common parasites of the native animals of the particular area.

11-22. *Angiostrongylus cantonensis. A. cantonensis* is normally a parasite that is found in the lungs of rats. It is enzootic in most areas of the Pacific. Snails, planarians, shrimp, and many other invertebrates serve as intermediate hosts. The intermediate hosts ingest the eggs of the parasite that are passed in the feces of the rat. Larvae hatch from the eggs and develop to the infective stage in the intermediate host. Rats eat the snails, releasing the larvae. The larvae make a peculiar migration during which they go to the brain for a period of development before they go to the lungs. Humans become infected in the same manner as rats, but the worms do not complete their migration to the lungs. They remain in the brain and spinal column, causing a disease known as *eosinophilic meningitis*. The worms develop to the adult stage in man, but they do not reach full maturity. Man is frequently infected in many of the Pacific islands, Vietnam, and Thailand. People who are infected have very severe headaches and other neurological symptoms. The spinal fluid is usually loaded with eosinophils. Occasionally immature worms are also recovered from the spinal fluid. There are other helminths that sometimes cause a similar syndrome; but in the areas where *A. cantonensis* is common among rats, it is the most frequent cause of eosinophilic meningitis in man. A skin test utilizing antigen made from the worm may be helpful in making a diagnosis.

11-23. *Anisakis. Anisakis* is a member of the *Ascaris* group. It is normally a parasite of fish-eating marine mammals. Little is known of the life history of the parasite, but it is thought that marine cephalopods serve as intermediate hosts and that marine fishes serve as paratenic hosts. Man becomes infected by eating insufficiently cooked marine fish. In man the larvae develop very little, but they burrow into the mucosa of the stomach or intestine. *Anisakis* infections are usually mistaken for peptic ulcers or malignant tumors. The parasite is most commonly reported in man from Japan. It is occasionally reported from Europe. You can expect to encounter *Anisakis* in any area where uncooked marine fishes are eaten.

11-24. *Lagocheilascaris. Lagocheilascaris minor* is normally a parasite of the intestinal tract of the cloudy leopard (feline). It has been reported to inhabit the tissues of the neck near the jaw, the nasal passages, and the maxillary sinuses of man. *L. minor* develops to maturity in man and even passes eggs which closely resemble *Toxocara* eggs. The worms are usually associated with a large abscess. You can recover both worms and eggs from the abscess exudates. The life cycle of the small worms (male 9 mm. long; female 15 mm. long) has not been worked out. Human infections have been reported from Tobago, Trinidad, and Surinam.

11-25. **Category III.** In this type of infection, humans are more or less normal, but unnatural, paratenic hosts. A paratenic host is a special type of transport host in which infective stage larvae persist without essential development and usually without growth. Beaver (1969) has proposed the term "paratenesis" to describe the various forms of transport involving paratenic hosts. These include transport through a series of paratenic hosts; transport through one or a series of paratenic hosts towards the final host; and transport from host to host, which preserves the infective larva from season to season (transport across time as well as across space).

11-26. *Gnathostoma spinigerum. G. spinigerum* is a parasite that lives in tumors within the walls of the stomachs of dogs, cats, and certain wild carnivores. The parasite is found in animals in Southeast Asia, Japan, China, India, Indonesia, parts of Europe, and Australia. Most human cases have been reported from Thailand.

11-27. Copepods serve as intermediate hosts. Coldblooded vertebrates, fish-eating birds, and mammals other than final hosts serve as paratenic hosts. Final hosts become infected when they ingest infected copepods or infected paratenic hosts. Paratenic hosts, including man, become infected when they ingest infected copepods or other infected paratenic hosts. The larva can pass through several paratenic hosts before it reaches a final host. In paratenic hosts, the parasite remains in a larval stage and becomes encapsulated in muscle tissue rather than maturing and migrating to the stomach wall.

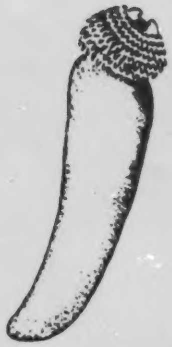


Figure 53. Larvae of *Gnathostoma spinigerum*.

11-28. The migrating larvae produce a kind of creeping eruption and occasionally cause serious problems by invading the eye. There is an eosinophilia associated with the infection, but that is a common finding with tissue-invading nematodes. The larvae must be removed from the skin for you to positively identify them. They measure 10 to 50 mm. by 1 to 2.5 mm., which is considerably larger than most nematode larvae that cause creeping eruption. The most distinctive feature is a large head bulb (fig. 53) that has eight rings of hooklets.

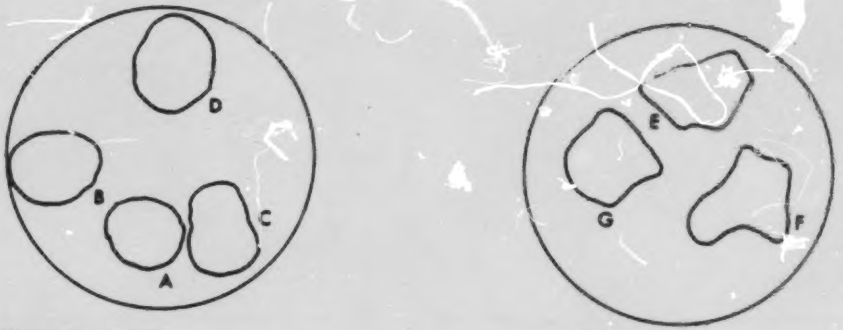
11-29. *Toxocara.* The genus *Toxocara* includes ascarids that infect dogs and cats. They are widely distributed over most of the world. Most birds and mammals, including man, can serve as paratenic hosts. In humans such infections are known as *Visceral larva migrans.* Young children about 1 to 4 years of age are most frequently infected. This is to be expected, since they put everything, including dirt, in their mouths.

11-30. As with *Ascaris lumbricoides,* the larvae migrate through the intestine and into the other body tissues; but instead of making the normal lung passage and returning to the intestine, *Toxocara* larvae continue to migrate through the body and concentrate particularly in the liver in humans. The resulting disease may be very mild, with an eosinophilia of 20 to 80 percent the only symptom noted. Frequently the disease is severe with intermittent fever, cough, hepatomegaly, and hyperglobulinemia. It is sometimes fatal. Specific diagnosis requires identification of the larvae, which are usually found in liver biopsies. Skin tests and serological procedures have shown some promise, but as yet they are not reliable.

11-31. *Ancylostoma.* Some species of the genus *Ancylostoma* cause the disease *cutaneous larva migrans.* The infective larvae penetrate the skin and migrate around, causing an intensely itching creeping eruption.

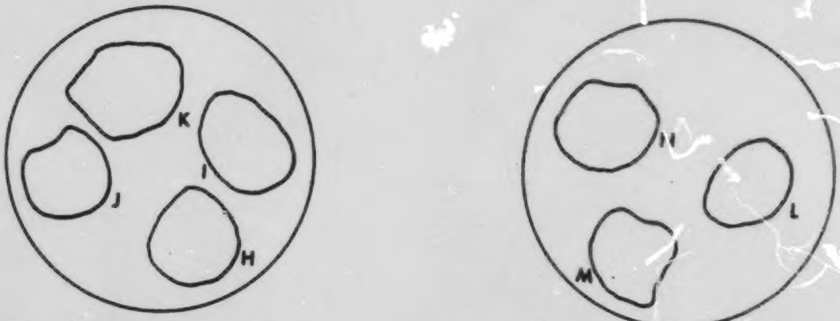
A diagnosis of cutaneous larva migrans is usually made by the physician on the appearance of the lesions.

AU-GAFS, AL (753901) 2100
*U.S. GOVERNMENT PRINTING OFFICE: 1975-645-814



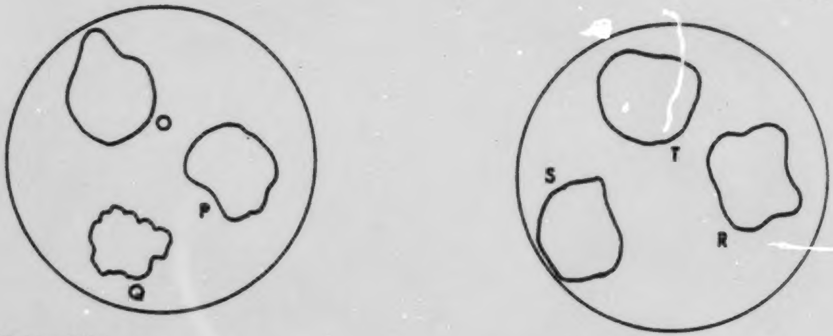
I. Young Trophozoites
A and B—Young ring forms.
C and D—Half-grown trophozoites.

II. Old Trophozoites
E, F, and G—Trophozoites with nuclear chromatin ready to subdivide.



III. Young Schizonts
H and I—Young Schizonts showing first division of nuclear chromatin.
J and K—Older schizonts showing from four to many subdivisions of nuclear chromatin.

IV. Half-grown Schizonts
L, M, and N—Schizonts showing seven, eight, and ten nuclear subdivisions.

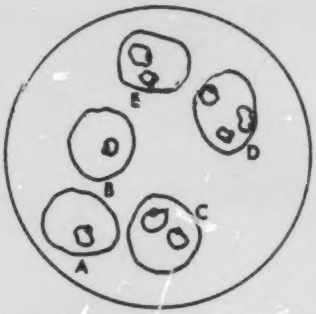


V. Mature Schizonts
O, P, and Q—Mature schizonts showing complete subdivision of nuclear chromatin and clumping of malarial pigment.

VI. Gametocytes or Sexual Forms
R—Male gametocyte or microgametocyte. Note diffuse nuclear chromatin.
S and T—Female gametocyte or macrogametocyte. Note compact chromatin.

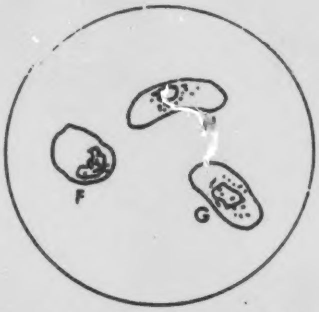
FOLDOUT 1 . . . .. . Plasmodiom Vivax

FOLDOUT 2 . . . . Plasmodium Falciparum



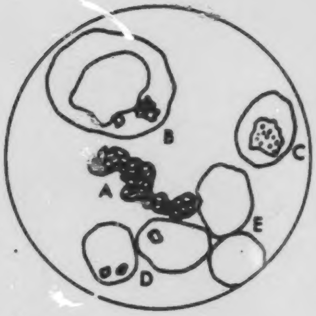
I. **Trophozoites in Peripheral Blood**

A—Young ring forms.
B, C, and D—Young trophozoites.
Note multiple infections of cells and appliqué forms.
E—These are oldest forms normally found in peripheral blood.



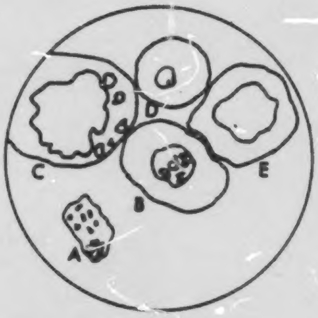
II. **Gametocytes or Sexual Forms in Peripheral Blood**

F—Gametocyte folded over.
G—Microgametocyte or male gametocyte. Note diffuse chromatin.
H—Female or macrogametocyte. Note compact chromatin.



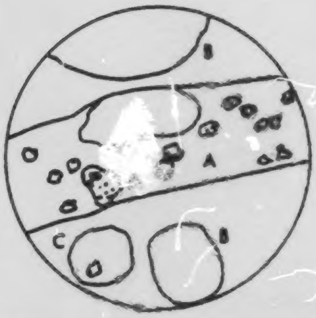
III. **Impression Smear of Spleen**

A—Free pigment of splenic pulp.
B—Pigment in macrophage.
C—Half-grown schizont in cell.
D—Parasitized red cells.
E—Lymphocytes.



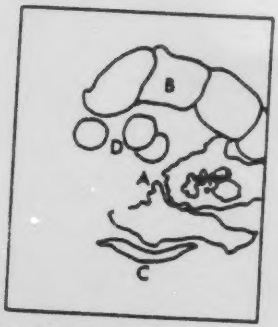
IV. **Impression Smear of Bone Marrow**

A—Free pigment granules.
B—Nucleated red cell.
C—Pigment in macrophage.
D—Parasitized red cell.
E—Eosinophile.



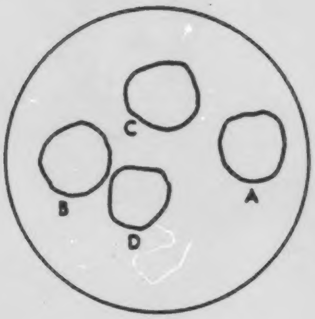
V. **Impression Smear of Brain**

A—Capillary blocked with parasitized erythrocytes.
B—Glial cells.
C—Trophozoite in red cell.
D—Maturing schizont.



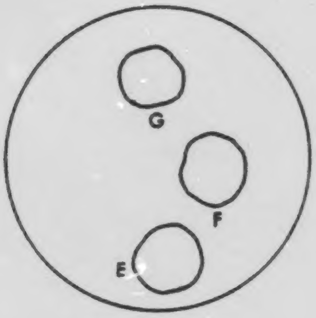
VI. **Section of Liver**

A—Küpffer cells with pigment.
B—Hepatic cells.
C—Endothelial cell.
D—Free red blood cells.



I. Young Trophozoite

A, B, and C—Progressively older ring forms.
D—Band trophozoite.

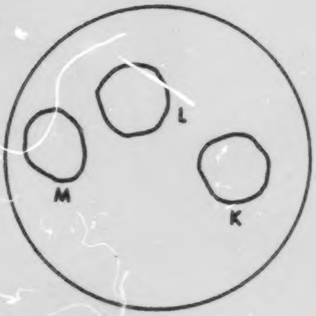


II. Half-grown Trophozoite

E—Ring form.
F and G—Mature trophozoites.
Note amount of pigment; compactness of cytoplasm.

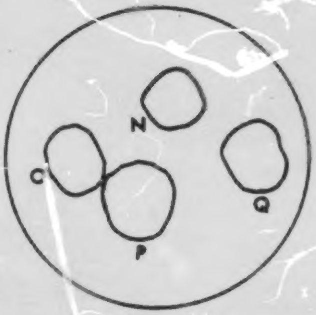
III. Young Schizonts

H—Band schizont.
I and J—Three- and five-nucleated schizonts.
Note large amount of pigment.



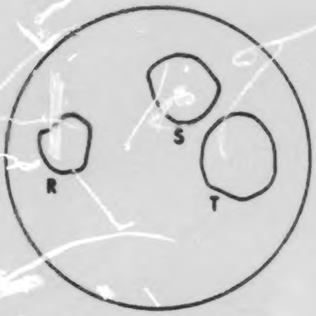
IV. Half-grown Schizonts

K, L, and M—Four- to six-nucleated schizonts. Note amount of pigment.



V. Mature Schizonts

N, O, P, and Q—Eight- or ten-nucleated schizonts ready to segment and release merozoites. Note "daisy" forms.



VI. Gametocytes or Sexual Forms

R and S—Male gametocytes or microgametocytes.
T—Female gametocyte or macrogametocyte.

NOTE: Color photographs in Foldouts 1, 2, and 3 could not be reproduced for inclusion in the "Trial Implementation of a Model System to Provide Military Curriculum Materials for use in Vocational and Technical Education."

10-3 371

90412 03 21

WORKBOOK

Clinical Parasitology

SELF-TEACHING AIDS
CRE
VRE
SRG

This workbook places the materials you need *where* you need them while you are studying. In it, you will find the Study Reference Guide, the Chapter Review Exercises and their answers, and the Volume Review Exercise. You can easily compare textual references with chapter exercise items without flipping pages back and forth in your text. You will not misplace any one of these essential study materials. You will have a single reference pamphlet in the proper sequence for learning.

These devices in your workbook are autoinstructional aids. They take the place of the teacher who would be directing your progress if you were in a classroom. The workbook puts these self-teachers into one booklet. If you will follow the study plan given in "Your Key to Career Development," which is in your course packet, you will be leading yourself by easily learned steps to mastery of your text.

If you have any questions which you cannot answer by referring to "Your Key to Career Development" or your course material, use ECI Form 17, "Student Request for Assistance," identify yourself and your inquiry fully and send it to ECI.

Keep the rest of this workbook in your files. Do not return any other part of it to ECI.

EXTENSION COURSE INSTITUTE
Air University

372

TABLE OF CONTENTS

Study Reference Guide

Chapter Review Exercises

Answers to Chapter Review Exercises

Volume Review Exercise

ECI Form No. 17

STUDY REFERENCE GUIDE

1. *Use this Guide as a Study Aid.* It emphasizes all important study areas of this volume.

2. *Use the Guide as you complete the Volume Review Exercise and for Review after Feedback on the Results.* After each item number on your VRE is a three digit number in parenthesis. That number corresponds to the Guide Number in this Study Reference Guide which shows you where the answer to that VRE item can be found in the text. When answering the items in your VRE, refer to the areas in the text indicated by these Guide Numbers. The VRE results will be sent to you on a postcard which will list the *actual VRE items you missed.* Go to your VRE booklet and locate the Guide Number for each item missed. List these Guide Numbers. Then go back to your textbook and carefully review the areas covered by these Guide Numbers. Review the entire VRE again before you take the closed-book Course Examination.

3. *Use the Guide for Follow-up after you complete the Course Examination.* The CE results will be sent to you on a postcard, which will indicate "Satisfactory" or "Unsatisfactory" completion. The card will list *Guide Numbers* relating to the questions missed. Locate these numbers in the Guide and draw a line under the Guide Number, topic, and reference. Review these areas to insure your mastery of the course.

Guide Number	*Guide Numbers 300 through 309*
300	Introduction to Medical Parasitology, pages 1-5
301	Introduction to Protozoa Infecting Man; Flagellates, pages 6-13
302	Amebae, pages 13-17
303	Sporozoa; Ciliates, pages 17-24
304	Introduction to Platyhelminthes Infecting Man; Trematodes (Flukes): Schistosomes (Blood Flukes), pages 25-30
305	Trematodes (Flukes): Liver Flukes; Intestinal Flukes; Lung Fluke (Paragonimus westermani), pages 30-34
306	Cestodes (Tapeworms), pages 34-39
307	Introduction to Nematodes Infecting Man; Intestinal Nematodes, pages 40-48
308	Filariae and Dracunculus, pages 48-52
309	Zoonotic Nematodes, pages 52-57

CHAPTER REVIEW EXERCISES

The following exercises are study aids. Write your answers in pencil in the space provided after each exercise. Immediately after completing each set of exercises, check your response against the answers for that set. Do not submit your answers to ECI for grading.

CHAPTER 1

Objective: To demonstrate a knowledge of the historical data pertaining to parasitic infections of man, the development of laboratory methods for the identification of parasites, classification of parasites, host-parasitic relationships, and the ill effects that parasitic infections have on man.

1. How long has man been subject to animal parasites? (Intro.-1)

2. In what country does the highest incidence of porkworm (*Trichinella spiralis*) occur? (Intro.-2)

3. What three factors are required for increasing an individual's ability and skill in finding and identifying human parasites? (Intro.-3)

4. What discovery late in the 17th century began a new era in parasitology? (1-2)

5. Which insect serves as an intermediate host for the human malaria parasite? (1-2)

6. Plasmodium falciparium, which has developed an even greater resistance to the prophylactic drugs routinely used, is a strain of what parasitic disease? (1-5)

375

7. The examination of what type of specimen provides the major workload in a parasitology laboratory? (1-8)

8. Name three of the phyla which are associated with the parasites of medical importance. (1-10; Table 1)

9. What kind of scientists are more properly involved when ectoparasites must be identified? (2-3)

10. What type of parasite infects humans with malaria? (2-2, 4)

11. Within what type of hosts are parasites capable of reproducing their own kind? (2-6)

12. A hog which is harboring the organism Trichinella spiralis serves as what type of host? (2-10)

13. What function is performed by vectors of parasites? (2-11)

14. What term is applied to the close association of two organisms of which parasitism is one aspect? (2-12)

15. List three ways in which parasite damage can be manifested in a host. (2-14)

16. What response by a human host may be caused by helminth (worm) invasion of the body's tissue? (2-17)

17. Name three modes of transmission by which parasites may gain access to the human body. (2-21)

18. What step in food preparation, if done inadequately, allows the food to become an unnecessary source of parasitic infection? (2-24)

CHAPTER 2

Objective: To demonstrate a knowledge of the major areas of geographical distribution, distinctive morphological features, methods of human invasion, possible pathological effects on man, and important aspects of laboratory identification for 23 protozoan parasites.

1. The life cycle of *Leishmania* may be divided into two stages. In one stage the parasite is called a leishmania-form organism. In what host does this stage of the parasite exist? (3-2)

2. In a second stage of the life cycle of *Leishmania*, the parasite is known as a flagellated leptomonas-form organism. In what insect vector does this stage of the parasite develop? (3-2)

3. If a technician prepares slide smears from a patient's peripheral blood to search for *Leishmania tropica* parasites, what would be the value of the laboratory examination? (3-8)

4. As the disease known as Espundia (caused by the *Leishmania braziliensis* parasites) advances, in what mucous membranes of man do the parasites localize? (3-9)

5. In what geographical areas is the disease caused by the parasite *Leishmania braziliensis* found? (3-9)

6. The disease mucocutaneous leishmaniasis (or Espundia), in its advanced stages, may destroy certain areas adjacent to the face. Name two areas which may be destroyed. (3-10)

7. List three laboratory procedures which may be used to assist in the identification of *Leishmania* parasites. (3-11)

8. On the continent of Africa there exists a disease commonly known as African or Rhodesian sleeping sickness. Which of the *Trypanosoma* parasites causes the disease? (3-12)

9. When performing laboratory diagnosis of *Trypanosoma rhodesiense*, one procedure involves preparation of a body fluid by centrifugation. What particular body fluid is centrifuged? (3-16)

10. Infection with Gambian disease appears milder than with the Rhodesian type, and symptoms include fever and headache recurring irregularly. Usually, would you consider Gambian disease to be a chronic or acute infection? (3-19)

11. What condition develops in patients who survive the acute phase of Chagas' disease? (3-23)

12. When making a laboratory examination of a fecal specimen from a patient with severe diarrhea, what is the predominant form of *Giardia lamblia* that you could expect to find? (3-27)

13. What is the most characteristic morphological structure of *Chilomastix mesnili*? (3-31)

14. Does the *Chilomastix mesnili* parasite cause any symptoms or illness in man? (3-32)

15. Give the approximate length and shape of the parasite found most often in the intestinal tract of man. (3-33, 35)

16. In an examination of vaginal specimens in females for *Trichomonas vaginalis*, one might possibly find a second species of *Trichomonas* due to possible contamination with feces. Name this species. (3-41)

17. In what part of the body would *Trichomonas tenax* be found if it should be present in man? (3-42)

18. *Trichomonas hominis* and *Trichomonas vaginalis*, when stained with hematoxylin, show a distinct differentiation from each other in their undulating membranes. How do the membranes differ? (3-48)

19. Which one of the *Entamoeba* parasites is the most pathogenic for man? (4-3)

20. One species of *Entamoeba* has a nucleus which is nearly perfectly circular. This circular nucleus is one important feature for identification of the species. Name the specific *Entamoeba*. (4-4)

21. What are the diagnostic characteristics that may usually be observed in the mature four-nucleate *Entamoeba histolytica* cyst? (4-6)

22. Which species of intestinal amebae is the largest? (4-10)

23. Name the *Entamoeba* which has the following features: In stained preparations the nucleus is very prominent, and on the inner periphery of the nuclear membranes the chromatin material is arranged in irregular masses. (4-11)

24. What symptoms and diseases are produced by *Entamoeba coli*? (4-15)

25. Laboratory examination of a tartar scraping specimen reveals the presence of *Entamoeba gingivalis*. What can be assumed concerning the condition of the person from whom the specimen was taken? (4-17, 18)

26. What are the important points by which you can recognize *Endolimax nana* trophozoites in stained preparations? (4-20)

27. Which species of amoeba quickly ruptures and disintegrates when in contact with tap water? (4-24)

28. Which intestinal amoeba of man has a single nucleus which persists throughout both the trophozoite and cystic stages of its life cycle? (4-27)

29. Is the life cycle of sporozoans sexual, asexual, or both? (5-2)

30. A fresh fecal specimen is taken from a person infected with *Isospora belli*. How would the immature oocyst be recognized? (5-7)

31. How would you recognize the *Toxoplasma gondii* organism when it is stained with Giesma? (5-10)

32. What disease-bearing parasites which can infect man are of the genus *Plasmodium?* (5-15)

33. Does the asexual cycle of the malarial parasite take place in man or in the mosquito? (5-18)

34. In which organ of man does the merozoite stage of the malarial parasite develop? (5-20)

35. During the reproductive cycle of the malarial parasite, what kind of daughters are developed by the schizonts? (5-20)

36. In what type of human blood cell does the trophozoite stage of the malarial parasite develop? (5-21)

37. List four species of malarial parasites which can infect man. (5-25)

382

38. Which species of malarial parasite is considered to be most widely distributed throughout the world? (5-26)

39. Which *Plasmodium* causes a disease which is sometimes called "tertian malaria," a term which refers to a 48-hour period required for the schizont development of the parasite to be completed? (5-26)

40. Of which *Plasmodium* species is the mature schizont or segimenter stage called "rosette" or "daisy" form? (5-32)

41. What types of smears should you use in searching for malarial parasites on stained peripheral blood smears? (5-37)

42. To stain peripheral blood smears for malarial parasites, should you use Wright's stain, Giemsa's stain, or both? (5-37)

43. What function is performed by the hair-like cilia of ciliated protozoa? (6-1)

44. In comparison with other protozoans which may infect man, what is the relative size of *Balantidium coli*? (6-2)

406

45. What symptoms or illness can be caused by *Balantidium coli*? (6-6)

CHAPTER [illegible]

Objective: To demonstrate a knowledge of the major areas of geographical distribution, distinctive morphological features, method of human invasion, possible pathological effects on man, and important aspects of laboratory identification for 17 flatworm parasites.

1. Which part of a tree or plant would you consider the trematode, or fluke, to resemble? (Intro.-1)

2. Tapeworms develop into proglottids. How could proglottids best be described? (Intro.-1)

3. Why are flotation techniques unacceptable for detecting the eggs of Platyhelminthes? (Intro.-4)

4. What names are given the trematodes that live in man's bloodstream? (7-2)

5. What is the most prominent feature which helps to identify the *Schistosoma mansoni* egg, and where on the egg is this feature located? (7-6)

6. In a specimen of what kind of material would you expect to find the characteristic eggs of *Schistosoma mansoni*? (7-8)

7. What is the comparative size of adult male and female *Schistosoma japonicum* parasites? (7-11)

8. Which species of Schistosomes causes schistosome hematuria? (7-15)

9. The egg of *Schistosoma haematobium* has a blunt-pointed spine. Where is this spine located on the egg? (7-18)

10. With recent infections caused by the *Schistosoma haematobium* parasite, what is the most frequent symptom noted? (7-19)

11. Laboratory technicians usually search for adult eggs in stool specimens of man to find most intestinal parasites in suspected cases. However, in case of a suspected infection with *Schistosoma haematobium*, what additional body specimen should be examined? (7-20)

12. How can you concentrate the eggs of *S. haematobium* in urine specimens collected from suspected cases of persons who may harbor the vesical blood fluke? (7-20)

13. Name the fluke which often causes an infection in man and is found mainly in sheep-raising countries. (7-22)

385

14. Man is a definitive host for the *Fasciola hepatica* species when infected with this parasite. What crustaceans are intermediate hosts for this species? (7-23)

15. What kind of specimen will you need if you have to positively differentiate the eggs of *Fasciola hepatica* from those of *Fasciolopsis buski?* (7-26)

16. In parts of China, Indochina, Japan, and Formosa, a certain fluke is considered the most important liver fluke in man. Name the fluke. (7-30)

17. What parasite, in very heavy infections, may produce fibrous tissue in the bile ducts that can result in cirrhosis of the liver? (7-33)

18. An egg specimen (taken from a person in northeastern Thailand) which is slightly smaller but similar to *Clonorchis* is very likely from which parasite? (7-36)

19. What species is sometimes called the giant intestinal fluke? (7-39)

20. List the approximate measurements of the species you selected in exercise 11. (7-40)

21. How do human infections occur in the transfer of the species you selected in exercise 11? (7-41)

22. For which parasite do snails of the Genus *Pironella* serve as first intermediate hosts? (7-44)

23. How do humans usually become infected with the *Metagonimus yokogawai* species? (7-49)

24. What is the name of the only lung fluke that infects man? (7-53)

25. Describe the shell of *Paragonimus westermani* eggs. (7-57)

26. By what means is the tapeworm attached to the intestinal wall of its host? (8-1)

27. Most parasitic tapeworms are provided with a cup-like sucker at each of four angles, and some species have an anterior *hook-bearing* portion. What name is given to this *hook-bearing* portion? (8-2)

28. Does a mature proglottid of a tapeworm have male, female, or combined male and female reproductive systems? (8-3)

29. What is the long name of the tapeworm which infects man and is commonly called the *fish* or *broad tapeworm*? (8-6)

30. What is the length of the *fish* or *broad tapeworm?* (8-6)

31. In both the mature and gravid proglottids of *Diphyllobothrium latum*, what are two principal diagnostic features that are centrally situated? (8-8)

32. Which is the largest of the tapeworms that infest man? (8-10)

33. In what animal meat would one expect to find the *T. saginata* species? (8-10)

34. What is the definitive host for the *T. saginata* parasites? (8-11)

35. Does *T. saginata* have either a rostellum or attachment hooks? (8-12)

36. How many lateral branches of the uterus may be found in a gravid proglottid of *T. saginata?* (8-12)

37. What is the importance of the size of *T. saginata* in relation to the pathology it can cause? (8-13)

38. How can the laboratory technician distinguish between *T. saginata* and *T. solium* parasites if he has one or more mature gravid proglottids from each of the two species? (8-14)

39. If the eggs of both *T. saginata* and *T. solium* are in a specimen for microscopic examination, what description would be used to report the presence of each species? (8-14)

40. How can the proglottids of the *Taenia* tapeworms be most easily observed? (8-14)

41. If the parasite *Taenia solium* has been acquired, what animal meat must have been consumed by the patient? (8-15)

42. Can the hog ingest the proglottids and ova of *Taenia solium* which have been excreted in the feces of a patient who has become infected? (8-16)

43. What disease is caused by ingestion of infective cysticercus larva? (8-16)

44. The head of the adult *Taenia solium* possesses a prominent rounded rostellum. With what arrangement of hooks is the rostellum armed? (8-17)

45. What is the relation of the width to the length of proglottids of *T. solium*? (8-17)

46. What is the maximum number of uterine branches possessed by *T. solium* in its gravid proglottids? (8-17)

47. What symptoms can *T. solium* adults in the small intestine of man cause? (8-18)

48. In respect to size, how would you consider *Hymenolepsis nana* to be classified? (8-19)

49. Which of the tapeworms found in America is the most common? (8-19)

50. What is the usual length of America's most common tapeworm? (8-19)

51. Name the definitive hosts for the *Hymenolepsis nana* species. (8-19)

52. Since the tapeworm *Hymenolepsis diminuta* is occasionally found in the intestine of man, why is it commonly referred to as the rat tapeworm? (8-24)

53. What is the maximum length of *Hymenolepsis diminuta*, and what is the maximum number of proglottids that it may have? (8-25)

54. How can the laboratory technician differentiate between *H.diminuta* and *H. nana* eggs? (8-25)

55. Why can the life cycle of *Hymenolepsis diminuta* not be completed in man? (8-26)

56. The species *Dipylidium caninum* is a common parasite of what two animals? (8-28)

57. Why are children most often infected with *D. caninum* parasite? (8-28)

58. With respect to length and number of proglottids, how do *D. caninum* and *H. diminuta* compare? (8-29)

59. What is the distinctive feature of the gravid proglottids of *D.* caninum? (8-29)

60. How much further development occurs when *D. caninum* eggs have been expelled by a cat or dog host? (8-30)

CHAPTER 4

1. What phylum includes the most common parasites of man? (Intro.-2)

2. On the basis of their habitat in the body, what are the two general kinds of nematodes? (Intro.-4)

3. Which intestinal nematode has an intermediate host in its life cycle? (9-1)

4. What *Enterobius vermicularis* egg characteristic allows them to attach readily to a person's clothing and perianal skin? (9-4)

5. How should you collect a specimen of *Enterobius vermicularis* eggs for making laboratory diagnosis? (9-7)

6. What color and shape are *Trichuris trichiura* (whipworm) eggs? (9-10)

7. Why may *Ascaris lumbricoides* remain viable in soil for months or even years? (9-13)

8. How do *Ascaris lumbricoides* eggs differ from hookworm eggs? (9-15)

9. Of the two species of hookworms of major importance to man, which is more likely to be found in the United States? (9-18)

10. How does the infective stage filariform larvae of *Necator americanus* infect the human host? (9-20)

11. If hookworm eggs are detected when you are performing a routine fecal examination using the zinc-sulfate centrifugal flotation method, what is the least number of egg-producing worms that may be infecting the patient? (9-22)

12. What is the sex of *Strongyloides stercoralis* in the parasitic stage? (9-26)

13. List two ways that you can differentiate the rhabditoid larvae of *Strongyloides stercoralis* from those of hookworms. (9-29)

14. The physician has a patient who ate some raw sausage yesterday. Today the patient is experiencing bloody diarrhea and other abdominal symptoms. You are able to find many *Trichinella spiralis* larvae in some of the leftover sausage, but you cannot find any larvae in a muscle biopsy specimen from the patient. Why? (9-32)

15. What two agglutination procedures are available and considered to be sensitive and adequately specific for *Trichinella spiralis* when the tests are performed on acute and convalescent serum? (9-34)

16. Where do microfilariae develop into infective third stage filariform larvae? (10-2)

17. How do the microfilariae of certain strains of *Wuchereria bancrofti* demonstrate nocturnal periodicity? (10-4)

18. When using the Knott technique of concentration to detect microfilariae, what is used to dilute 2 cc. of venous blood? (10-7)

19. How can the microfilariae of *Brugia malayi* and *Wuchereria bancrofti* be differentiated? (10-9)

20. When do the microfilariae of *Loa loa* appear in the peripheral circulation? (10-11)

21. How do black flies obtain microfilariae of *Onchocerca volvulus* when they take a blood meal? (10-13)

22. If *Dipetalonema perstans* is considered to be harmless, why is it necessary that you be able to recognize the microfilariae of this species? (10-16)

23. How must skin snips be made if the microfilariae of *Dipetalonema streptocerca* are most likely to be detected? (10-17)

24. If a patient is suspected of being infected by *Mansonella ozzardi*, how can microfilariae be recovered for laboratory examination? (10-18)

25. What is the comparative size of the adult female *Dracunculus medinensis* and the filarial worms? (10-20)

26. If a patient has blisters on the skin that are suspected to be caused by *Dracunculus medinensis*, how can the larvae be demonstrated? (10-24)

27. When a human is infected with an animal parasite, what is the resulting infection called? (11-1)

28. What is the name of the only intestinal capillariid known to parasitize man? (11-4)

29. To make a positive diagnosis of *Capillaria hepatica*, a biopsy of what kind of tissue must be made? (11-7)

30. In what stage can *Ancylostoma ceylonicum* be differentiated from *Ancylostoma duodenale?* (11-8)

31. List two features of *Temidens deminutus* which distinguish it from a hookworm. (11-9)

32. When examining sputum and feces specimens, what may be mistaken as *Syngamus laryngeus* eggs? (11-10)

33. What would you do to differentiate hookworm eggs from eggs of *Trichostrongylus orientalis?* (11-13)

34. How can you diagnose an infection caused by *Thelazia californiensis?* (11-14)

35. When examining a feces specimen, you may confuse *Physaloptera caucasica* eggs with what other kind of eggs? (11-15)

36. What kind of laboratory specimen may be found to contain giant kidney worm eggs? (11-16)

37. What portion of man's anatomy may be infected by *Gongylonema pulchrum?* (11-17)

38. To what phylum of parasites do the thorny-headed worms belong? (11-18)

39. When coin lesions are discovered on radiographic examinations of the human lung, what parasite infection may be suspected? (11-20)

40. How does the rat lung worm (*Angiostrongylus cantonensis*) produce the disease eosinophilic meningitis in man? (11-22)

41. What parasite burrows into the mucosa of the stomach or intestine and causes an infection that may be mistaken for peptic ulcers or malignant tumors? (11-23)

42. What parasite may develop to maturity in the tissues of the neck near the jaw, the nasal passage, and the maxillary sinuses of man, and passes eggs which resemble those of *Toxocara?* (11-24)

43. What relation exists between a paratenic host and the larvae of a parasite? (11-25)

44. What is the most distinctive feature of *Gnathostoma spinigerum* found in man? (11-28)

45. Larva of what genus may frequently cause an infection in young children that is known as visceral larva migrans? (11-29)

46. What disease characterized by an itching, creeping skin eruption is caused by some species of *Ancylostoma?* (11-31)

ANSWERS FOR CHAPTER REVIEW EXERCISES

CHAPTER 1

1. From the earliest recorded history, man has been subject to animal parasites. (Intro.-1)
2. The highest incidence of porkworm *(Trichinella spiralis)* occurs in the United States. (Intro.-2)
3. Three factors required for increasing an individual's ability and skill in finding and identifying human parasites are:
   a. Individual initiative.
   b. Technical knowledge.
   c. Experience.
   (Intro.-3)
4. The discovery of the microscope late in the 17th century began a new era in parasitology. (1-2)
5. The mosquito serves as an intermediate host for the human malaria parasite. (1-2)
6. Malaria. (1-5)
7. The examination of fecal specimens provides the major workload in a parasitology section. (1-8)
8. Protozoa, Platyhelminthes, Nematoda, Acanthocephala, Annelida, and Arthropoda. (1-10; Table 1)
9. Entomologists. (2-3)
10. Endoparasites. (2-2, 4)
11. Definitive hosts. (2-6)
12. A reservoir host. (2-10)
13. A vector transfers parasites from one host to another. (2-11)
14. Symbiosis. (2-12)
15. Trauma, lytic action, tissue response, blood loss, and secondary infection. (2-14)
16. The human host will produce eosinophilia. (2-17)
17. Parasites may gain access to the human body by means of:
   a. Food.
   b. Soil or water.
   c. Filth.
   d. Arthropods.
   (2-21)
18. Cooking. (2-24)

CHAPTER 2

1. In both man and other mammals. (3-2)
2. The sandfly. (3-2)
3. Such an examination is useless, because the parasites are found in the tissues adjacent to the sores which they produce, rather than in the peripheral blood. (3-8)
4. In those of the nose and pharnyx. (3-9)
5. In large areas of tropical America, from Yucatan in Mexico through Central America, and into northern Argentina. (3-9)

6. The nasal septum, or the surfaces of the palate. (3-10)
7. *a.* Examination of exudates or biopsies collected from involved areas.
   b. Cultures.
   c. Serological procedures.
   (3-11)
8. *Trypanosoma rhodesiense* and *Trypanosoma gambiense.* (3-12)
9. Spinal fluid. (3-16)
10. Chronic. (3-19)
11. They develop the disease in its chronic state. (3-23)
12. The trophozoite. (3-27)
13. There is a proturberance on one margin of *Chilomastix mesnili* which gives the cysts the shape of a lemon. (3-31)
14. No, the *Chilomastix mesnili* parasite is not pathogenic. (3-32)
15. It is twice the diameter of a red blood cell in length, and its shape resembles a longitudinally cut pear. (3-33, 35)
16. It is possible for *Trichomonas hominis*, found in fecal material, to accidentally contaminate vaginal secretion specimens. (3-41)
17. In the mouth, around the teeth and gingival margins. (3-42)
18. *Trichomonas hominis* has a longer, undulating membrane. (3-48)
19. *Entamoeba histolytica.* (4-3)
20. *Entamoeba histolytica.* (4-4)
21. Four nuclei, rod-like chromatoidal bodies, and bead-like chromatin granules. (4-6)
22. *Entamoeba coli.* (4-10)
23. *Entamoeba coli* (4-11)
24. *Entamoeba coli* does not produce symptoms nor pathology in man. (4-15)
25. Because of poor oral hygiene, the person probably has diseased gums. (4-17, 18)
26. Nuclear characteristics, size, and appearance. (4-20)
27. *Dientamoeba fragilis.* (4-24)
28. *Iodamoeba butschlii.* (4-27)
29. Both – one phase of the cycle is sexual and the other phase is asexual. (5-2)
30. The immature oocyst would appear as a thick-walled, lemon-shaped, transparent structure containing a single spherical mass of protoplasm. (5-7)
31. The organism shows a delicate, light blue cytoplasm with a reddish, oval nucleus towards the broad end. (5-10)
32. Malaria parasites. (5-15)
33. In man. (5-18)
34. In the liver. (5-20)
35. Merozoites. (5-20)

36. In a red blood cell. (5-21)

37. The four species of malarial parasites that can infect man are ***Plasmodium vivax, Plasmodium malariae, Plasmodium falciparum***, and ***Plasmodium ovale.*** (5-25)

38. ***Plasmodium vivax.*** (5-26)

39. ***Plasmodium vivax.*** (5-26)

40. ***Plasmodium malariae.*** (5-32)

41. Both thick and thin preparations. (5-37)

42. For staining peripheral blood smears for malarial parasites, you should use both Wright's stain and Giemsa's stain. (5-37)

43. The cilia provide the organism with locomotion. (6-1)

44. *Balantidium coli* is the largest protozoan which may infect man. (6-2)

45. *Balantidium coli* can cause diarrhea, and has in some cases even caused death. (6-6)

CHAPTER 3

1. The leaf of a tree or plant. (Intro.-1)

2. Proglottids could best be described as chain-like segments. (Intro.-1)

3. The specific gravity of the eggs is too high for them to be consistently floated. (Intro.-4)

4. Schistosomes or blood flukes. (7-2)

5. A long and sharp lateral spine that is located along one side on about the posterior third of the egg. (7-6)

6. Fecal material. (7-8)

7. The female is slightly longer, about 26 mm. as compared with the 12 to 20 mm. length of the male. (7-11)

8. *Schistosoma haematobium.* (7-15)

9. At its posterior end. (7-18)

10. Blood in the urine of the patient. (7-19)

11. Urine specimens. (7-20)

12. By centrifugation. (7-20)

13. *Fasciola hepatica.* (7-22)

14. Snails. (7-23)

15. A specimen of uncontaminated bile. (7-26)

16. *Clonorchis sinensis.* (7-30)

17. *Clonorchis sinensis.* (7-33)

18. *Opisthorchis viverrini.* (7-36)

19. *Fasciolopsis buski.* (7-39)

20. *Fasciolopsis buski* adult parasites measure over an inch in length and about a half inch in width. (7-40)

21. In man, infections with *F. buski* occur when cracking nuts of the water chestnut or water caltrop with the teeth. (7-41)

22. *Heterophyes heterophyes.* (7-44)
23. By eating uncooked freshwater fish on which the infective melacercariae have encysted. (7-49)
24. *Paragonimus westermani.* (7-53)
25. The shell is relatively thick and golden brown in color. (7-57)
26. The tapeworm attaches itself to the intestinal wall of its host by means of its hooked head or *scolex.* (8-1)
27. A hook-bearing portion at the anterior end of the tapeworm is given the name *rostellum.* (8-2)
28. Each mature proglottid has both male and female reproductive systems. (8-3)
29. *Diphyllobothrium latum.* (8-6)
30. The *Diphyllobothrium latum,* or *fish* or *broad* tapeworm, has a length up to 35 feet. (8-6)
31. The rosette-shaped uterus and the genital pore. (8-8)
32. *Taenia saginata.* (8-10)
33. Beef. (8-10)
34. Man. (8-11)
35. The head of *T. saginata* has neither a rostellum nor attachment hooks. (8-12)
36. From 15 to 20. (8-12)
37. Because of its large size, the *T. saginata* species is often the cause of excessive digestive pathology. (8-13)
38. By counting the number of lateral uterine branches or arms. (8-14)
39. The eggs of the *T. saginata* species cannot be distinguished from those of *T. solium* by microscopic examination, so only the presence of eggs of the Genus *Taenia* could be reported. (8-14)
40. Press them between two microscopic slides and use a hand lens. (8-14)
41. Improperly cooked pork. (8-15)
42. Yes. (8-16)
43. Cysticercosis. (8-16)
44. A double circle of hooks. (8-17)
45. The mature proglottids of *T. solium* are slightly wider than long. (8-17)
46. Thirteen. (8-17)
47. Irritation to the mucosa, intestinal obstruction, and nervous disorders due to the production of toxic substances. (8-18)
48. As the dwarf tapeworm of man. (8-19)
49. *Hymenolepsis nana.* (8-19)
50. About 2 inches. (8-19)
51. Man, rats, and mice. (8-19)
52. Because of the frequency of finding it in the small intestines of rats. (8-24)
53. Twenty inches, with up to 2,000 proglottids. (8-25)
54. The eggs of *H. diminuta* are larger and lack the polar filaments which are characteristic of *H. nana.* (8-25)

55. The life cycle requires a stage of development in one of various arthropods. (8-26)
56. The dog and the cat. (8-28)
57. Because of their frequent close contact with infected cats and dogs as pets. (8-28)
58. They are of the same approximate length, but *D. caninum* usually has fewer proglottids. (8-29)
59. They resemble the shape of a cucumber or pumpkin seed. (8-29)
60. The eggs must be ingested by one of various insects, such as the dog louse or certain larval fleas, which serve as intermediate hosts. (8-30)

CHAPTER 4

1. Nematodo. (Intro.-2)
2. Intestinal and somatic or tissue roundworms. (Intro.-4)
3. *Trichinella.* (9-1)
4. The outer shell of the egg is sticky. (9-4)
5. Press the sticky side of clear scotch tape to the perianal folds of the patient and then stick the tape to a glass slide. (9-7)
6. *Trichuris trichiura* eggs are golden brown and barrel shaped. (9-10)
7. Because they are very resistant to drying and low temperatures. (9-13)
8. *Ascaris lumbricoides* eggs have a much thicker shell; and they contain an undivided cell mass, whereas hookworm eggs contain a zygote that is in the four or eight cell stage. (9-15)
9. *Necator americanus.* (9-18)
10. By penetrating the skin. (9-20)
11. One or two. (9-22)
12. Female. (9-26)
13. (1) The buccal canal of *Strongyloides* is very short, while that of the hookworm is longer and narrow; and (2) the genital primordium of *Strongyloides* is much larger than in the hookworm. (9-29)
14. It takes 7 to 14 days after exposure for most of the larvae to be filtered out in the muscle. (9-32)
15. Bentonite flocculation test and indirect hemagglutination test. (9-34)
16. In appropriate bloodfeeding insects. (10-2)
17. They circulate in peripheral blood mainly at night. (10-4)
18. Use 10 cc. of 2 percent formalin. (10-7)
19. The microfilariae of *Brugia malayi* have two cells in the tip of the tail which are not present in *Wuchereria bancrofti.* (10-9)
20. Mainly during the daytime. (10-11)
21. Black flies have chewing mouthparts; therefore, it is necessary for them to chew a hole through the skin to the capillary bed and then lap up the blood as it pools up in the hole. The microfilariae escape from the skin in the tissue fluids and are taken up with the blood. (10-13)
22. You will need to be able to differentiate them from the microfilariae of the more harmful species. (10-16)
23. They must be made deep enough to include dermal tissue. (10-17)

24. By taking a sample of peripheral blood. (10-18)
25. The adult female *Dracunculus medinensis* is much larger. (10-20)
26. By placing the blister in contact with water and allowing the blister to rupture. (10-24)
27. A zoonotic infection. (11-1)
28. *Capillaria philippinensis.* (11-4)
29. Liver. (11-7)
30. In the adult stage. (11-8)
31. The buccal capsule of *Ternidens deminutus* is terminally located, and the buccal cavity is guarded on the inside by a double row of stiff bristles. (11-9)
32. Pollen grains. (11-10)
33. Measure the eggs; they should measure 70 to 90 by 40 to 50 microns, while hookworm eggs measure up to 70 by 40 microns. (11-13)
34. The nematode parasite is found in the conjunctival sac. After it is removed from the eye, examine the worm. It has a very rough cuticle, and it measures about 4.5 mm. by 0.25 to 0.85 mm. (11-14)
35. *Ascaris* eggs. (11-15)
36. Urine. (11-16)
37. The mucosa and subdermal connective tissues in the vicinity of the mouth. (11-17)
38. *Acanthocephala.* (11-18)
39. *Dirofilaria immitis.* (11-20)
40. The larvae make a migration through the brain and spinal column, where they develop into adult worms before settling in the lungs of rats. In man they also migrate to the brain and spinal column; but they remain there, producing damage which causes the symptoms of eosinophilic meningitis. (11-22)
41. *Anisakis.* (11-23)
42. *Lagocheilascaris minor.* (11-24)
43. The infective stage larvae persist within a paratenic host without essential development and usually without growth. (11-25)
44. The larva has a large head bulb that has eight rings of hooklets. (11-28)
45. *Toxocara.* (11-29)
46. Cutaneous larva migrans. (11-31)

STOP- 1. MATCH ANSWER SHEET TO THIS EXERCISE NUMBER. 2. USE NUMBER 1 PENCIL.

90412 03 21

VOLUME REVIEW EXERCISE

Carefully read the following:

DO'S

1. Check the "course," "volume," and "form" numbers from the answer sheet address tab against the "VRE answer sheet identification number" in the righthand column of the shipping list. If numbers do not match, take action to return the answer sheet and the shipping list to ECI immediately with a note of explanation.

2. Note that numerical sequence on answer sheet alternates across from column to column.

3. Use only medium sharp # 1 black lead pencil for marking answer sheet.

4. Circle the correct answer in this test booklet. After you are sure of your answers, transfer them to the answer sheet. If you *have* to change an answer on the answer sheet, be sure that the erasure is complete. Use a clean eraser. But try to avoid any erasure on the answer sheet if at all possible.

5. Take action to return entire answer sheet to ECI.

6. Keep Volume Review Exercise booklet for review and reference.

7. If *mandatorily* enrolled student, process questions or comments through your unit trainer or OJT supervisor.
   If *voluntarily* enrolled student, send questions or comments to ECI on ECI Form 17.

DON'T

1. Don't use answer sheets other than one furnished specifically for each review exercise.

2. Don't mark on the answer sheet except to fill in marking blocks. Double marks or excessive markings which overflow marking blocks will register as errors.

3. Don't fold, spindle, staple, tape, or mutilate the answer sheet.

4. Don't use ink or any marking other than with a # 1 black lead pencil.

Note: The 3-digit number in parenthesis immediately following each item number in this Volume Review Exercise represents a Guide Number in the Study Reference Guide which in turn indicates the area of the text where the answer to that item can be found. For proper use of these Guide Numbers in assisting you with your Volume Review Exercise, read carefully the instructions in the heading of the Study Reference Guide.

Multiple Choice

Chapter 1

1. (300) Which of the following statements concerning parasitology is *not* true?
   - a. Parasitology deals with the relationship between a parasite and its host.
   - b. The discovery of the microscope began a new era in parasitology.
   - c. A parasite derives its sustenance from another organism without making compensation.
   - d. The major developments in modern parasitology came in the 20th century.

2. (300) In recent years, which of the following malaria parasites has developed the most resistance to treatment?
   - a. *Plasmodium falciparum.*
   - b. *Plasmodium malariae.*
   - c. *Plasmodium ovale.*
   - d. *Plasmodium vivax.*

3. (300) Which of the following is *not* a principal phylum of medically significant parasites?
   - a. Sporozoa.
   - b. Arthropoda.
   - c. Protozoa.
   - d. Platyhelminthes.

4. (300) The parasitic infections which are the most important health problems in the tropics today are borne by
   - a. soil or water.
   - b. food.
   - c. arthropod vectors.
   - d. filth.

5. (300) Which insect serves as the definitive host for the human malaria parasite?
   - a. Fly.
   - b. Mosquito.
   - c. Tick.
   - d. Snail.

6. (300) Which of the following foods offers the greatest possibility for parasitic infection of man?
   - a. Fried fish.
   - b. Raw crayfish.
   - c. Boiled shrimp.
   - d. Fried bacon.

7. (300) All of the following are aspects of symbiosis *except*
   - a. mutualism.
   - b. commensalism.
   - c. parasitism.
   - d. trauma.

8. (300) For a parasite to exist, all of the following conditions are necessary *except*
   - a. a suitable host.
   - b. benefit to both host and parasite.
   - c. a dependable means of transmission to the host.
   - d. environmental conditions providing suitable access to the host.

Chapter 2

9. (301) During one stage in the life cycle of *Leishmania donovani*, the parasite lives as a flagellated leptomonas-form organism. Within which of the following insect vectors does this occur?
   - a. Bees.
   - b. Mosquitoes.
   - c. Sandflies.
   - d. Ticks.

10. (301) Which of the following countries has the highest infection rate with the parasite *Leishmania braziliensis?*

   a. India.
   b. South America.
   c. Northern Europe.
   d. North America.

11. (301) When patients survive the acute phase of the disease resulting from infection with *Trypanosoma cruzi*, what condition usually develops?

   a. Freedom from infection.
   b. A chronic state of the disease.
   c. Re-occurring acute attacks.
   d. Secondary bacterial infection.

12. (301) One procedure which is *not* used for laboratory diagnosis of *Trypanosoma rhodesiense* is

   a. spinal fluid preparation.
   b. demonstration in sternal bone marrow aspirates.
   c. complement fixation.
   d. stained blood smears.

13. (301) Which choice is correct of Gambian sleeping sickness?

   a. Requires a vector.
   b. Usually fatal.
   c. Usually diagnosed by complement fixation.
   d. Usually more serious than the Rhodesian type.

14. (301) The presence of flagellates in the genitourinary tract of a female patient strongly suggests a diagnosis of

   a. *Entamoeba coli.*
   b. *Trichomonas tenax.*
   c. *Trichomonas hominis.*
   d. *Trichomonas vaginalis.*

15. (302) Which of the following is *true* concerning *Entamoeba coli?*

   a. It moves in a definite direction.
   b. It is found only in South America.
   c. Its nucleus is usually invisible.
   d. It produces neither symptoms nor pathology in man.

16. (302) Of the amebae discussed in the text, how many inhabit the human intestinal tract?

   a. Four.
   b. Five.
   c. Six.
   d. Seven.

17. (302) All of the following diagnostic characteristics may usually be observed in the mature *Entamoeba histolytica* cyst *except*

   a. four nuclei.
   b. glycogen vacuoles.
   c. rod-like chromatoidal bodies.
   d. bead-like chromatin granules.

18. (302) All of the following statements relative to *Entamoeba gingivalis* are true *except* that

   a. its presence may indicate poor oral hygiene.
   b. it thrives on diseased gums.
   c. it is a pathogenic type of ameba.
   d. it is associated with the tartar between the teeth.

19. (301) All of the following laboratory procedures may be used to assist in the identification of *Leishmania* parasites *except*

a. cultures.
b. serological methods.
c. examination of exudates.
d. examination of peripheral blood.

20. (301) Which of the following does the *Gardia lamblia* trophozoite most resemble in shape?

a. A longitudinally cut pear.
b. A sliced watermelon.
c. An orange.
d. A lemon.

21. (303) Malaria can produce all of the following symptoms *except*

a. anemia.
b. enlargement of the liver.
c. congestion of the kidneys.
d. enlargement of the heart.

22. (302) With a drop in temperature, the *Dientamoeba fragilis* will

a. become active.
b. become encysted.
c. degenerate.
d. flatten out.

23. (301) A feature by which *Trichomonas hominis* and *Trichomonas vaginalis* can be differentiated is that

a. the undulating membrane of *Trichomonas hominis* is longer.
b. the undulating membrane of *Trichomonas hominis* is shorter.
c. *Trichomonas vaginalis* forms a genuine cyst.
d. *Trichomonas hominis* forms a genuine cyst.

24. (303) Organisms of the subphylum Sporozoa have an interesting life cycle in their development. Which, if any, phases do they undergo during their development?

a. Sexual only.
b. Asexual only.
c. Both sexual and asexual.
d. Neither sexual nor asexual.

25. (303) When stained with Giemsa, the color of the cytoplasm of the *Toxoplasma gondii* organism appears

a. grey.
b. brown.
c. yellow.
d. light blue.

26. (302) The important points by which the *Endolimax nana* can be recognized in stained preparations are all of the following *except*

a. size.
b. appearance.
c. the round karyosome body.
d. nuclear characteristics.

27. (303) Of the various species of malaria, the one which causes the most severe disease is *Plasmodium*

a. *ovale.*
b. *vivax.*
c. *malariae.*
d. *falciparum.*

28. (303) Which of the following species of malarial parasites is most widely distributed throughout the world?

a. *Plasmodium malariae.*
b. *Plasmodium ovale.*
c. *Plasmodium vivax.*
d. *Plasmodium falciparum.*

29. (303) Which of the following is *not* true of fevers seen in malaria?

a. They are rhythmic.
b. They coincide with the release of mature gametocytes.
c. The period between peaks varies according to which species of parasites causes the infection.
d. They coincide with the rupture of red blood cells.

30. (302) The only species of intestinal amebae of man in which a single nucleus persists throughout both the trophozoite and cystic stages is the *Iodamoeba*

a. *coli.*
b. *fragilis.*
c. *butschlii.*
d. *histolytica.*

31. (303) In which organ of man does the exoerythrocytic stage of the malarial parasite begin?

a. Stomach.
b. Bladder.
c. Kidney.
d. Liver.

32. (303) As compared with other protozoa, *Balantidium coli* is

a. the largest found in man.
b. found in invertebrate animals only.
c. the smallest found in man.
d. found in vertebrate animals only.

33. (303) Where in man does the erythrocytic stage of the malarial parasite take place?

a. Liver.
b. Red blood cells.
c. Salivary glands.
d. White blood cells.

Chapter 3

34. (304) What is the incubation period of cercariae of *Schistosoma mansoni?*

a. 1 to 2 weeks.
b. 3 to 4 weeks.
c. 5 to 6 weeks.
d. 7 to 8 weeks.

35. (304) The egg of the *Schistosoma haematobium* has a blunt-pointed spine. Where is this spine located?

a. Within the egg.
b. Along one side of the egg.
c. At the anterior end.
d. At the posterior end.

36. (305) The only way that *Fasciola hepatica* can be positively identified is to recover typical eggs from

a. feces.
b. urine.
c. sputum.
d. uncontaminated bile.

37. (304) What species of fluke causes the disease known as intestinal schistosomiasis?

a. *Schistosoma mansoni.*
b. *Schistosoma japonicum.*
c. *Schistosoma haematobium.*
d. *Fasciola hepatica.*

38. (305) Humans normally become infected with *Metagonimus yokogawai* as a result of eating

a. uncooked fresh water fish.
b. pickled German sausage.
c. undercooked pork.
d. snails.

39. (305) How may false fascioliasus be ruled out?

a. Provide a lettuce-free diet for 3 days or more.
b. Provide a watercress-free diet for 3 days or more.
c. Provide a liver-free diet for 3 days or more.
d. Provide a lamb-free diet for 3 days or more.

40. (305) Which one of the following is called the giant intestinal fluke of man?

a. *Fasciola buski.*
b. *Fasciola hepatica.*
c. *Schistosoma japonicum.*
d. *Opisthorchis sinensis.*

41. (304) In what way are schistosomes unique among flukes of man?

a. Each individual worm has both male and female organs.
b. The males and females are separate worms.
c. Their intermediate host is snails.
d. They develop as single unsegmented units.

42. (305) Pathological effects of *Fasciolopsis buski* parasites on the human host include all of the following *except*

a. hoven.
b. anemia.
c. emaciation.
d. ulceration of intestinal mucosa.

43 (304) Which of the following species of Schistosoma causes schistosomal hematuria (blood in urine)?

a. *Schistosoma haematobium.*
b. *Schistosoma mansoni.*
c. *Schistosoma japonicum.*
d. *Schistosoma sinensis.*

44. (306) The *Dipylidium caninum* species is the most common tapeworm found in the

a. dog and cat.
b. dog and pig.
c. cat and pig.
d. pig and horse.

45. (305) In very heavy infections of man with *Metagonimus yokogawai*, all of the following symptoms may be present *except*

a. diarrhea.
b. cirrhosis of the liver.
c. cardiac disturbance.
d. persistent intestinal disturbances.

46. (304) With one fluke egg you have studied, a wart-like thickening may be seen near the posterior end when the egg is positioned properly. Which of the following has this feature?

a. *Schistosoma japonicum.*
b. *Schistosoma mansoni.*
c. *Schistosoma haematobium.*
d. *Fasciola hepatica.*

47. (304) In comparing *Schistosoma japonicum* with *Schistosoma mansoni*, which of the following statements is true?

a. The adult worms of *Schistosoma japonicum* inhabit the large intestine.
b. Their life cycles are essentially the same.
c. The adult worms of *Schistosoma mansoni* inhabit the small intestine.
d. The eggs of *Schistosoma japonicum* are larger than those of *Schistosoma mansoni.*

48. (306) The gravid proglottid of the *Taenia solium* possesses approximately how many lateral branches on each side of the uterine tube?

a. 3 to 5.
b. 5 to 7.
c. 7 to 13.
d. 10 to 17.

49. (306) The gravid proglottid of the *Taenia saginata* possesses approximately how many main lateral branches on each side of the uterine stem?

a. 3 or more.
b. 5 or more.
c. 10 or more.
d. 15 or more.

50. (305) What common Far Eastern liver parasite causes damage ranging from minor inflammation and lesions to cirrhosis of the liver and death?

a. *Fasciola hepatica.*
b. *Clonorchis sinensis.*
c. *Fasciolopsis buski.*
d. *Heterophyes heterophyes.*

51. (306) In comparison with the ova and worm of *Hymenolepsis nana,* what is the size of the ova and worm of *Hymenolepsis diminuta?*

a. The ova is smaller and the maximum length of the worm is greater.
b. The ova is larger and the maximum length of the worm is greater.
c. The ova is the same size and the maximum length of the worm is less.
d. The size of the ova and the maximum length of the worms are the same.

52. (306) The *Diphyllobothrium latum* (commonly known as the fish or broad tapeworm) may have a length up to

a. 5 feet.
b. 35 feet.
c. 5 inches.
d. 35 inches.

53. (305) Diagnosis of paragonimiasis, caused by the *Paragonimus westermani* parasite, is based on finding the characteristic eggs in all of the following *except*

a. sputum.
b. feces.
c. urine.
d. pleural aspirates.

54. (306) The adult tapeworm is attached to the intestinal wall of its host by its

a. tail.
b. proglottid.
c. scolex (head).
d. proglottid and head.

55. (306) Which of the following is known as the dwarf tapeworm of man and is the most common tapeworm found in America?

a. *Hymenolepsis diminuta.*
b. *Hymenolepsis nana.*
c. *Taenia saginata.*
d. *Taenia solium.*

Chapter 4

56. (307) What is the most common nematode infecting humans in the United States?

a. *Trichuris trichiura.*
b. *Enterobius vermicularis.*
c. *Ancylostoma duodenale.*
d. *Trichinella spiralis.*

4

57. (307) When rhabditiform larvae of the *Strongyloide stercoralis* follow the indirect route of development, they

a. develop into free-living adult male and female worms.
b. live for as long as 2 months in the soil.
c. develop into infective filariform larvae within 24 hours after leaving the intestine.
d. have usually been deposited on dry, cool soil.

58. (308) The microfilariae of *Onchocerca volvulus* are usually found in what type of specimen?

a. Fecal specimen collected at night.
b. Gastric contents collected at any time.
c. Peripheral blood specimen collected at peak of fever.
d. Skin-snip specimen collected at any time.

59. (307) *Trichinella* is unique among intestinal nematodes of man in that

a. its life cycle does not include any developmental stages outside the body of a host.
b. the young larvae live and grow in involuntary muscle.
c. its principal reservoir is the rat.
d. it has an embryonic stage known as microfilaria.

60. (308) Concerning *Brugia malayi* and *Wuchereria bancrofti*, which choice correctly presents a differentiating characteristic?

a. Adult *Brugia malayi* are about twice the size of *Wuchereria bancrofti*.
b. Adult *Brugia malayi* are about half as large as *Wuchereria bancrofti*.
c. The microfilariae of *Brugia malayi* are sheathed.
d. The microfilariae of *Wuchereria bancrofti* have two cells in the tip of the tail.

61. (307) Which of the following statements correctly describes the relationship between *Necator americanus* and *Ancylostoma duodenale* parasites?

a. The eggs cannot be differentiated.
b. *Ancylostoma duodenale* are more slender.
c. *Necator americanus* are larger.
d. Only *Necator americanus* eggs are referred to as "hookworm ova."

62. (308) From which of the following may microfilariae of *Mansonella ozzardi* be recovered?

a. Dermal tissue.
b. Urine.
c. Liver.
d. Peripheral blood.

63. (307) Which of the following points should be noted to differentiate the rhabditoid larvae of *Strongyloides* from those of hookworms?

a. The larva of hookworm has a notched tail.
b. The larva of *Strongyloides* has a pointed tail.
c. The buccal canal of *Strongyloides* is very short.
d. The genital primordium of *Strongyloides* is much smaller.

64. (308) How do the microfilariae of certain strains of *Wuchereria bancrofti* demonstrate nocturnal periodicity?

a. They circulate in peripheral blood mainly at night.
b. They circulate in peripheral blood mainly during the day.
c. They are present in the feces only at night.
d. They are present in the feces only during the day.

65. (307) After exposure, how many days does it take for most the larvae of *Trichinella spiralis* to reach striated muscle?

a. 1 to 2 days.
b. 3 to 5 days.
c. 7 to 14 days.
d. 60 to 90 days.

66. (309) In what stage can *Ancylostoma ceylanicum* be differentiated easily from *Ancylostoma duodenale?*

a. Egg.
b. Adult.
c. Larval.
d. Microfilariae.

67. (307) Why may *Ascaris lumbricoides* eggs remain viable in soil for years?

a. They are unsegmented when passed.
b. They become infective in a minimum of 2 months.
c. They have thin inner shells.
d. They are resistant to drying and low temperatures.

68. (308) If a patient is suspected of being infected by *Dracunculus medinensis,* how may the larvae be demonstrated?

a. Rupture any blisters by applying water.
b. Centrifuge arterial blood.
c. Examine fecal material microscopically.
d. Prepare egg cultures.

69. (309) Which of the following descriptions would be useful in confirming a diagnosis of *Trichostrongylus orientalis* infection?

a. Eggs are smaller than hookworm eggs.
b. Eggs are more blunt on both ends than hookworm eggs.
c. Eggs measure 65 by 35 microns.
d. Eggs measure 90 by 50 microns.

70. (309) Within what kind of host do infective stage larvae persist without essential development and usually without growth?

a. Inadequate.
b. Intermediate.
c. Paratenic.
d. Final.

71. (307) Eggs of *Ascaris lumbricoides* differ from hookworm eggs in that *Ascaris lumbricoides* eggs

a. have much thinner shells.
b. contain an undivided cell mass.
c. develop to the infective stage in from 5 to 8 days.
d. contain a zygote in the four- or eight-cell stage.

72. (307) Which of the following may be used effectively to collect a specimen of *Enterobius vermicularis* eggs for making laboratory diagnosis?

a. Clear Scotch tape.
b. A centrifuge.
c. A test tube.
d. A syringe.

73. (309) What infection of young children may be caused by parasites of the genus *Toxocara?*

a. *Cutaneous larva migrans.*
b. Ringworm.
c. *Visceral larva migrans.*
d. Infantile paralysis.

74. (309) The course of their migrations leads the larvae of *Angiostrongylus cantonensis* (rat lungworm) to what vital organ in both rats and man?

a. Lungs.
b. Liver.
c. Kidneys.
d. Brain.

75. (308) The microfilariae of *Loa loa* demonstrate what type of periodicity?

a. Aperiodicity.
b. Nocturnal.
c. Variable.
d. Diurnal.

76. (309) Which of the following statements is *not* true concerning parasites of the genus *Thelazia californiensis?*

a. They have been reported from several oriental countries.
b. Certain species of flies serve as intermediate hosts.
c. They normally inhabit the conjunctival sac of dogs.
d. They are incidental parasites of man.

77. (309) What portion of man's anatomy is usually infected by *Gongylonema pulchrum?*

a. Subdermal tissues near the mouth.
b. Mucosa of the esophagus.
c. The peripheral blood.
d. The tissues of the eye.

78. (309) *Ancylostoma* has been proved to be a cause of

a. massive abcesses.
b. *cutaneous larva migrans.*
c. elephantiasis.
d. *visceral larva migrans.*

79. (309) When a human is infected with an animal parasite, the infection is called

a. enzootic.
b. paratenic.
c. zoonotic.
d. intermediate.

80. (309) Which of the following statements describes Category II of the categories of relationships existing among zoonotic helminth infections?

a. Humans are normal and adequate, though unnatural, final hosts.
b. Humans are more or less normal, but unnatural, paratenic hosts.
c. Humans are more or less normal, but inadequare, final hosts.
d. Humans are more or less normal, but unnatural, intermediate hosts.

415

90412 04 0570 0973

CDC 90412

MEDICAL LABORATORY TECHNICIAN - MICROBIOLOGY

(AFSC 90470)

Volume 4

Laboratory Procedures in Clinical Mycology

10-3

Extension Course Institute
Air University

Preface

THIS IS the last volume of Course 90412, *Medical Laboratory Technician - Microbiology*. In this volume you will learn some of the laboratory procedures used in clinical mycology. This will include the characteristics of fungi such as taxonomic relationships, cellular morphology, cultural properties, and the fungi as disease agents. You will also learn collection and processing techniques, as well as how to ship specimens. There are discussions of yeastlike fungi, monomorphic molds, dimorphic fungi, and saprophytic fungi. Finally, the last chapter of the volume sets forth general guidelines for the collection, preservation, packaging, and shipment of clinical virological specimens.

Three oversize illustrations are numbered as foldouts 1, 2, and 3. These foldouts are printed as a separate inclosure to this volume.

If you have questions on the accuracy or currency of the subject matter of this text, or recommendations for its improvement, send them to Tech Tng CEN (MSSTW), Sheppard AFB, TX 76311.

If you have questions on course enrollment or administration, or on any of ECI's instructional aids (Your Key to Career Development, Study Reference Guides, Chapter Review Exercises, Volume Review Exercise, and Course Examination), consult your education officer, training officer, or NCO, as appropriate. If he can't answer your questions, send them to ECI, Gunter AFB, Alabama 36114, preferably on ECI Form 17, Student Request for Assistance.

This volume is valued at 30 hours (10 points).

Material in this volume is technically accurate, adequate, and current as of August 1969.

Contents

Page

Preface iii

Chapter

1 INTRODUCTION TO MEDICAL MYCOLOGY 1
2 THE YEASTLIKE FUNGI AND THE MONOMORPHIC MOLDS 15
3 THE PATHOGENIC DIMORPHIC FUNGI 43
4 THE SAPROPHYTIC FUNGI 53
5 VIROLOGY 70

Bibliography 93

Glossary 94

Introduction to Medical Mycology

FUNGUS—THAT'S THE stuff that grows on the north side of trees! Perhaps this is the usual thought we have when we hear this word. However, this is not entirely true. There are many, many different types of fungi; some are harmless or even beneficial, others deadly. Beneficial—in that we like certain species on our steaks (mushrooms)—deadly—some are capable of invading the body and slowly involving every organ system and structure.

2. Medical mycology is the study of fungi which can invade the human body and produce disease. Fungi are small members of the plant kingdom without chlorophyll, roots, stems, or leaves. They have extremely simple fundamental structures known as hyphae (vegetative structures) and spores (reproductive structures). With these two basic structures they live, multiply, and carry out the other functions characteristic of microorganisms in general.

3. The first chapter of this volume will show the basis for classifying fungi, describe their morphological and cultural properties, and give methods for the collection and processing of specimens. The techniques commonly used in identification of the medically important fungi will be discussed. We will cover in some detail in subsequent chapters the yeastlike fungi, the monomorphic molds, the dimorphic or diphasic fungi, and finally, the saprophytic forms. Chapter 5 of this volume introduces the student to the nature of the viruses and sets forth the guidelines for collection, preservation, and shipment of clinical specimens for diagnostic testing.

1. Characteristics of the Fungi

1-1. In recent years botanists have re-examined some of the traditional bases for the classification of plants. Fungi are no longer considered to be derived from or related to the algae. Phylum Thallophyta, which included both the algae and the fungi, has been eliminated. The bacteria, fungi, and slime molds are now taxonomically segregated into their own respective phyla. The fungi are members of the Phylum Eumycophyta or "true fungi" of the plant kingdom.

1-2. **Taxonomic Relationships.** There are four classes of fungi, as shown in figure 1, differentiated primarily by type of hypha and spore and mode of reproduction. The distinguishing characteristics of each class are summarized in table 1. The terms used to describe the various structural parts are defined in the glossary at the end of the volume. The class *Phycomycetes* contains the most primitive of the fungi. Most members produce broad, aseptate hyphae. They reproduce asexually by forming sporangia that contain sporangiospores (endospores). Sexual reproduction, when present, is by means of gametes, gametangia, oospores, or zygospores. The asexual structures are depicted in figure 2. The class Ascomycetes is represented by two morphologically distinct types. The first type has unicellular, round, or oval forms reproducing asexually by the simple budding of blastospores, as shown in figure 3,A. This type is represented by the perfect yeast, genus *Saccharomyces*. Under favorable conditions, sexual ascospores are formed. Four or eight ascospores develop within each saclike inclosure called an ascus. The asci break open to release the ascospores. This is illustrated in figure 3,B. Note in figure 3,C, that a second type of *Ascomycetes* has septate hyphae producing filamentous forms which reproduce asexually by spores called conidia.

1-3. In the class *Basidiomycetes* the hyphae is septate. Sexual reproduction is by means of basidiospores developed on club-shaped structures called basidia. Each basidium usually bears four exogenous basidiospores resembling toes on a foot. Figure 4 shows these characteristics. The class *Deuteromycetes* (fungi imperfecti) contains, with few exceptions, the fungi pathogenic to man. These fungi lack a demonstrable means of sexual reproduction and, therefore, are considered "imperfect." They are represented by two morphologically distinct types: a septate, filamentous (mold) form, shown in figure 5, and an imperfect yeast

TABLE 1

CHARACTERISTICS OF THE TAXONOMIC CLASSES OF FUNGI

Taxonomic class of Fungi	*Hypha*	*Type of Reproduction*	*Characteristic Spore*	*Origin of Spore*	*Examples of Fungi*	*Pathogenicity*
Phycomycetes	Aseptate	Asexual	Sporangiospore	Sporangiospore	Nuisance fungi incluing genera *Absidia*. *Mucor*, and *Rhizopus*	--Very rare--- Mucormycosis
		Sexual	Zygospore or oospore	Fussion of nuclei		
Ascomycetes	Septate	Asexual	Blastospore	Budding	*Allescheria* *Aspergillus* *Piedraia*	----Rare---- Maduromycosis Aspergillosis Black Piedra
			Conidium	Conidiophore		
		Sexual	Ascospore	Ascus	*Saccharomyces* (perfect yeast)	
Basidiomycetes	Septate	Sexual	Basidiospore	Basidium	Mushrooms, smuts and rusts	-----Rare---- Mushroom poisoning
Deuteromycetes (fungi imperfecti	Septate	Asexual	Thallospore	Thallus (hypha)	Most saprophytes and pathogens encountered in medical mycology (Imperfect mold and yeast)	Most mycoses encountered in medical mycology
			Conidium	Conidiophore		

- KINGDOM—Plant
  - SUBKINGDOM—Thallophyta
    - PHYLUM—Eumycophyta
      - SUPER CLASS—Eumycetes
        - CLASS:
          - Phycomycetes
          - Ascomycetes
          - Basidiomycetes
          - Deuteromycetes

Figure 1. Classification of fungi.

form resembling the perfect yeast form, shown in figure 3,A.

1-4. **Cellular Morphology.** The microscopic morphology of the fungi shown in the foregoing figures is an important aid to identification. The type of hypha, spore and spore-bearing structure, and the manner in which these organs relate to each other in many instances will give a direct, accurate diagnosis of the mycotic agent under investigation. When direct morphological studies in themselves are not sufficient, special techniques must be utilized. These techniques will be discussed in detail in Section 3 of this chapter.

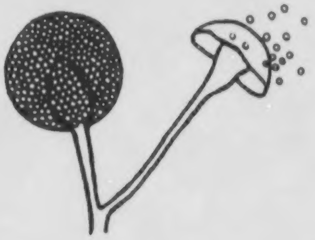


Figure 2. Asexual structures of Phycomycetes.

1-5. The life cycle of the fungi can be separated into two phases: the vegetative and the reproductive. The *vegetative* phase is characterized by elongated, branching filaments which may be septate or aseptate (refer to the glossary for the distinction between them). These tubular filaments are known individually as hypha, or as "hyphae," when more than one is being considered. A mass of hyphae is referred to as a mycelium. The hyphae have cell walls of varying thickness lined with a layer of cytoplasm. The thickness of these walls may increase greatly as the fungus matures. Chitin is the primary constituent of the cell walls of most of the higher fungi; while in some forms, cellulose is probably the chief ingredient. If cross-walls (septa) are formed, the hypha is divided into cells, and each cell may contain one, two, or several nuclei. When cross-walls are not formed (aseptate or coenocytic hypha), the entire hyphal filament is made up of a single cell with many nuclei embedded in the peripheral cytoplasm and scattered fairly uniformly throughout its mass.

1-6. Vegetative hyphae have a twofold function. They first serve to anchor the fungus to the substratum—either by penetration of simple hyphal filaments, or as in the case of the *Genus Rhizopus,* by means of rootlike rhizoids which permit better adherence to the substratum and provide a greater surface area for direct diffusion. The diffusion of enzymes from the fungus into the substratum converts nutrient material into simpler compounds which can be absorbed by the fungus and used for its growth and reproduction. The second important function of vegetative hyphae, then, is the procurement of food.

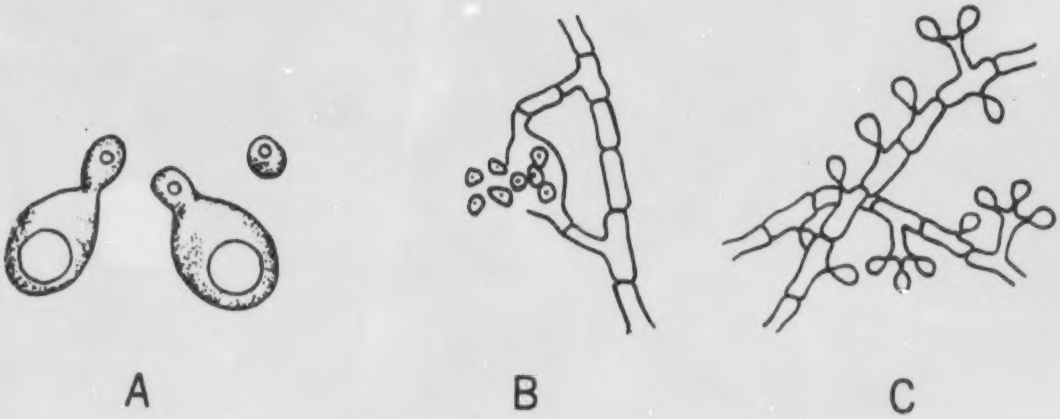


Figure 3. Reproductive forms of Ascomycetes.

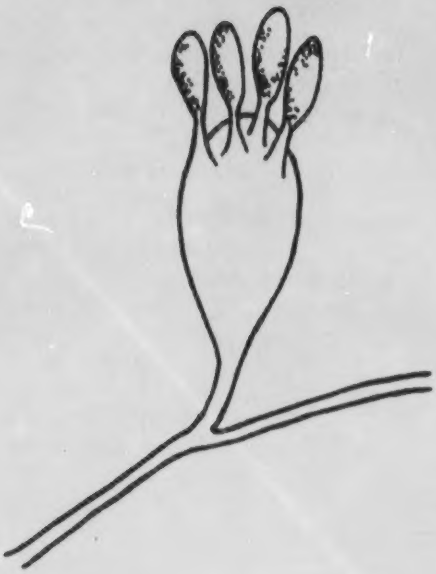


Figure 4. Characteristics of basidiospores.

1-7. The *reproductive* phase usually commences when the fungus has reached maturity, or when it faces unfavorable environmental conditions, such as temperature variations or lack of nourishment. The reproductive organ is called a spore. The spore may be either unicellular or multicellular; and when released from the mother plant, it is capable of duplicating the species. Although primarily reproductive in function, spores additionally serve to disperse the fungus and protect it from extinction during adverse environmental conditions. Spores may be sexual (formed by the fusion of two nuclei) or asexual. Asexual sporulation takes place by simple fission of a single nucleus. Some fungi are capable of only asexual sporulation, whereas others have the capacity for both asexual and sexual spore formation. Along with characteristic morphology, the way in which they reproduce is an important means of classifying and identifying fungi.

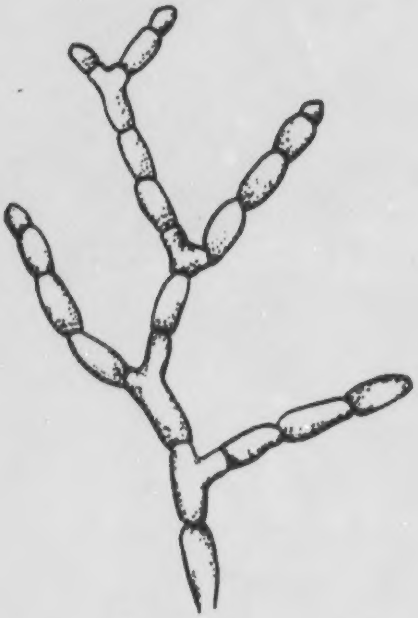


Figure 5. Mold form of Deuteromycetes.

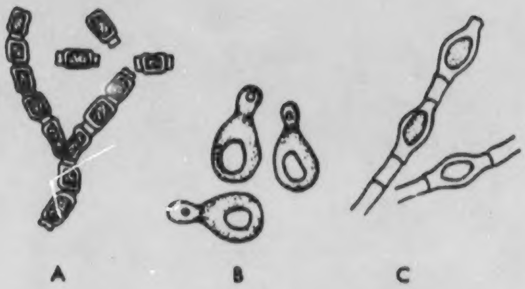


Figure 6. Varieties of thallospores.

1-8. There are three principal types of asexual spores: the thallospore (body spore), endospore, and the ectospore. The thallospores are further delineated into three varieties: the arthrospore, blastospore, and chlamydospore. These various forms are shown in figure 6. Arthrospores develop within a septate hyphal filament, shown in figure 6,A. They are disseminated by a breaking up of the spore chain thus formed. Blastospores, shown in figure 6,B, arise from simple budding of the yeast or yeastlike fungi. They may also develop directly from true or pseudomycelia. The chlamydospore, shown in figure 6,C, is a rounded, thick-walled body which is formed by a vegetative hyphal segment. It is a highly resistant, resting spore capable of survival long after the vegetative mycelium has lost its viability.

1-9. A second asexual type of spore, called an endospore (see fig. 2), is borne within a saclike

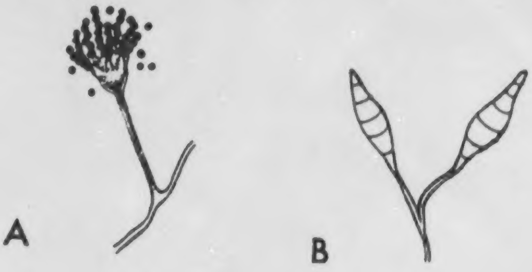


Figure 7. Ectospores (microconidium, macroconidium).

TABLE 2

CLASSIFICATION OF PATHOGENIC FUNGI ACCORDING TO MACROSCOPIC MORPHOLOGY

THE YEAST-LIKE FUNGI

Superficial Fungus

- *Trichosporon cutaneum*

Cutaneous Fungi

- *Candida albicans*[1] and *Candida* spp.

Systemic Fungi

- *Cryptococcus neoformans*
- *Geotrichum candidum*

MONOMORPHIC MOLD FUNGI

Superficial Fungi

- *Piedraia hortai*
- *Pullularia werneckii*

Cutaneous (Dermatophytic) Fungi

- *Microsporum* spp.
- *Trichophyton* spp.
- *Epidermophyton floccosum*
- *Keratinomyces ajelloi*

Subcutaneous (Chromoblastomycotic) Fungi

- *Cladosporium carrionii*
- *Fonsecaea compacta*
- *Fonsecaea pedrosoi*
- *Phialophora verrucosa*

Subcutaneous (Maduromycotic) Fungi

- *Allescheria boydii*
- *Madurella grisea*
- *Madurella mycetomii*
- *Phialophora jeanselmei*

Systemic Fungi

- *Actinomyces israelii*
- *Actinomyces bovis*
- *Nocardia asteroides*
- *Coccidioides immitis*

DIMORPHIC FUNGI

Subcutaneous Fungi

- *Sporotrichum schenckii*

SYSTEMIC FUNGI

- *Blastomyces dermatitidis*
- *Paracoccidioides brasiliensis*
- *Histoplasma capsulatum*

---

[1] *Candida albicans* has been implicated in systemic infections.

TABLE 3

CLASSIFICATION OF PATHOGENIC FUNGI ACCORDING TO MYCOSIS

Mycosis	*Important Etiological Agents*	*Macroscopic Morphology*
SUPERFICIAL		
Black Piedra	*Piedraia hortai*	Monomorphic Mold.
Tinea Nigra	*Pullularia werneckii*	Monomorphic Mold.
Tinea Versicolor	*Malassezia furfur*	Unknown to date.
White Piedra	*Trichosporon cutaneum*	Monomorphic Yeast.
CUTANEOUS		
Candidiasis	*Candida albicans* and other *Candida* spp	Monomorphic Yeast.
Tinea Barbae	*Trichophyton* spp	Monomorphic Mold.
Tinea Capitis	*Microsporum* spp	Monomorphic Mold.
	Trichophyton spp	Monomorphic Mold.
Tinea Corporis	Same as Tinea Capitis	
Tinea Cruris	*Candida albicans*	Monomorphic Yeast.
	Epidermophyton floccosum	Monomorphic Mold.
	Trichophyton spp	Monomorphic Mold.
Tinea Pedis	Same as Tinea Cruris	
SUBCUTANEOUS		
Chromoblastomycosis	*Cladosporium carrionii*	Monomorphic Mold.
	Fonsecaea compacta	Monomorphic Mold.
	Fonsecaea pedrosoi	Monomorphic Mold.
	Phialophora verrucosa	Monomorphic Mold.
Mycetoma, Actinomycotic	*Actinomyces israelii*	Monomorphic Mold.
	Nocardia asteroides	Monomorphic Mold.
	Nocardia brasiliensis	Monomorphic Mold.
Mycetoma, Maduromycotic	*Allescheria boydii*	Monomorphic Mold.
	Phialophora jeanselmei	Monomorphic Mold.
	Madurella grisea	Monomorphic Mold.
	Madurella mycetomii[4]	Monomorphic Mold.
	Cephalosporium falciforme	Monomorphic Mold.
Rhinosporidiosis	*Rhinosporidium seeberii*	Unknown to date.
Sporotrichosis	*Sporotrichum schenckii*	Dimorphic.
SYSTEMIC		
Actinomycosis	*Actinomyces israelii*	Monomorphic Mold.
Blastomycosis	*Blastomyces dermatitidis*	Dimorphic.
Candidiasis	*Candida albicans* and other *Candida* spp	Monomorphic Yeast
Coccidioidomycosis	*Coccidioides immitis*	Monomorphic Mold.
Cladosporiosis	*Cladosporium bantianum*	Monomorphic Mold.
Cryptococcosis	*Crytococcus neoformans*	Monomorphic Yeast.
Geotrichosis	*Geotrichum candidum*	Monomorphic Yeast.
Histoplasmosis	*Histoplasma capsulatum*	Dimorphic.
Nocardiosis	*Nocardia asteroides*	Monomorphic Mold.
	Nocardia brasiliensis	Monomorphic Mold.
Paracoccidioidomycosis	*Paracoccidiodes brasiliensis*	Dimorphic

membrane. The last variety of asexual spore, the ectospore, is formed either directly on the mycelium or on a modified supportive structure (e.g., conidiophore, sporangiophore). Ectospores are always borne free (no inclosure), vary greatly in size and shape, and are technically referred to as conidia. The small, unicellular type is called a microconidium, while the larger, multicellular type is termed a macroconidium. Examples of both types are shown in figure 7.A and B. Almost all the medically important fungi belong to the class *Deuteromycetes*, in which no sexual means of reproduction has been found. Therefore, our discussion of sexual sporulation will be limited to those few cases where specific identification demands it.

1-10. **Cultural Properties.** Like bacteria, the fungi are lacking in chlorophyll and must rely on some source of available organic material for food. There are fungi which can produce enzymes capable of breaking down nearly all the organic substances known to man. In some cases, their food requirements include complex sugars, proteins, and fats. Other fungi have little need for preformed nutrients and can grow in pickle brine or organic acids when the necessary minerals are present. It is not unusual to find fungi growing on fingerprints left on camera lenses. Unlike the bacteria, which prefer a slightly alkaline pH, fungi grow best at an acid pH, normally around pH 5.6. Commercially prepared Sabouraud's dextrose agar, the primary isolation medium for most pathogenic fungi, will closely approximate this figure when rehydrated for use. A modified Sabouraud's dextrose agar with pH 6.5 to 6.8 and containing antibiotics has been found even more effective in recoving medically important fungi on initial isolation. With the exception of members of the genus *Actinomyces,* the pathogenic fungi are aerobic and require oxygen for growth.

1-11. Since the fungi and many bacteria can use identical substances for growth, antibiotics (chloramphenicol, cycloheximide) are often incorporated into Sabouraud's dextrose agar to inhibit contaminating bacteria and saprophytic fungi. There is one disadvantage, however; numerous fungi of medical importance are also inhibited and may not be recovered from initial plating if antibiotics are present.

1-12. There is a considerable difference in the growth rate between the fungi and the bacteria. When culturing bacteria, colony examination and biochemical testing can usually be accomplished 24 to 48 hours after the specimen is inoculated to media. The fungi, particularly the pathogenic forms, may require 3 to 5 weeks, or longer, to reach a growth state which permits definitive diagnosis.

1-13. From the standpoint of size, the fungi are relatively large in comparison to the bacteria. The yeasts (nonfilamentous fungi) generally reach a diameter of about 5 or 6 microns, almost as large as an average red blood cell. Filamentous mold hyphae normally range in width from 5 to 50 microns and may attain a length of several millimeters.

1-14. **Colony Morphology.** One of the most important criteria for identifying the fungi is colonial appearance. As previously noted, there are two basic fungal forms: the yeasts (characterized by simple budding) and the filamentous molds (recognized by production of innumerable filamentous hyphae). Certain fungus species of medical importance are known to multiply only by budding, regardless of whether they are incubated at 25° C. or 37° C. This budding type of multiplication produces a pasty or mucoid, rounded colony on the substratum that could easily be mistaken for bacterial growth. Fungi which propagate only by budding are designated *monomorphic* (one form) *yeasts.*

1-15. Other genera of medical importance are known to multiply solely by the protrusion of a germ tube through the spore wall (spore germination), whether cultured at 25° C. or 37° C. These germ tubes become the long filaments, or hyphae, and they ultimately form the mycelial mass characteristic of molds. Mycelial growth is usually quite rapid, and macroscopic differentiation from the yeast form presents no great problem. The aerial hyphae of the mycelium usually give rise to spores which are disseminated by air currents to propagate the species. These filamentous forms that reproduce only by spore germination are defined as the *monomorphic* (one form) *molds.*

1-16. Another group of fungi, including those which cause some of the most serious mycotic infections in man, possess the unique capability of multiplying at 37° C. in the form of the monomorphic yeasts and at 25° C. in the form of the monomorphic molds. By exhibiting this temperature-dependent diphasic phenomenon, a given species of the group can exist either as a saprophytic mold in nature or as a parasitic yeast form in human tissues. These morphological variations are reversible when incubation temperatures are adjusted, and many times this reversibility assists in accurate diagnosis. Those fungi capable of existing in two forms are known as the *dimorphic* (two-form) *fungi.* Foldout 1, detail A, (Separate inclosure) shows typical colonies of dimorphic fungi, and table 2 lists representative species of each group.

1-17. **Fungi as Agents of Disease.** When a person is afflicted with a mycotic infection, the condition is normally referred to as a mycosis. Fungi can be categorized as to the type of tissue for which they have a natural affinity. The four categories are as follows;

- Superficial mycosis.
- Cutaneous mycosis.
- Subcutaneous mycosis.
- Systemic mycosis.

1-18. The etiological agents of the *superficial mycoses* are confined to the outermost layers of the skin and the hair. The superficial mycoses are generally the least serious of the fungus diseases. The agents of the *cutaneous mycoses,* sometimes called the dermatophytes, possess a particular affinity for the keratin of the skin, hair, and nails. They cause infections of a more serious nature than the superficial fungi, including ringworm infections of the feet, groin, or nails.

1-19. The responsible agents of the *subcutaneous mycoses* invade muscle tissue. These mycotic infections are considerably more serious than the cutaneous type. The agents of the *systemic mycoses* attack the deep tissues and organs of the body, often creating symptoms that resemble other diseases of the particular organ or tissue invaded. It is not unusual for a person so afflicted to demonstrate cutaneous manifestations, as well. The deep-seated fungal infections are the most serious encountered in medical mycology. An outline of

TABLE 4
MICROSCOPIC APPEARANCE OF PATHOGENIC FUNGI IN HAIR INFECTIONS

Microscopic Morphology	*Fungi*	Mycosis
Black, hard, nodules or granules (mycelium) firmly attached to hair shaft; asci with ascospores present within nodules.	*Piedraia hortai*	Black Piedra, a superficial mycosis
White, tan to light brown, soft nodules (mycelium), loosely attached to hair shaft; arthrospores and blastospores may be seen.	*Trichosporon cutaneum*	White Piedra, a superficial mycosis
Mycelium and arthrospores within (endothrix) hair shaft.	*Trichophyton soudanense* *Trichophyton tonsurans* *Trichophyton violaceum* *Trichophyton yaoundei*	Tinea, a cutaneous mycosis (endothrix hair invasion)
Mycelium within hair shaft, mycelium secondarily breaks out to surface of hair shaft; arthrospores formed only on outside (ectothrix) of hair shaft.	*Microsporum audouinii* *Microsporum canis* *Microsporum distortum* *Microsporum ferrugineum* *Microsporum gypseum* *Trichophyton equinum* *Trichophyton megninii* *Trichophyton mentagrophytes* *Trichophyton rubrum* *Trichophyton verrucosum*	Tinea, a cutaneous mycosis (ectothrix hair invasion)
Mycelium within hair shaft; no arthrospores formed; hair shaft usually contains fat droplets and empty channels resulting from disintegrating hyphae.	*Trichophyton schoenleinii*	Tinea, a cutaneous mycosis (favic-endothrix hair invasion)

the pathogenic fungi according to type of mycosis is given in table 3. The type of morphism characteristic of the etiological agents is also included.

2. Collection and Processing of Specimens

2-1. In medical mycology, clinical laboratory procedures are directed toward the demonstration, isolation, and identification of pathogenic fungi found in body tissue and fluids. Ordinarily, only simple, inexpensive equipment is required. The site of infection will dictate the type of specimen that can be obtained. For the most part, specimens received or collected by the mycology laboratory will be skin, nail scrapings, or suspect hairs. However, it is well to remember that the systemic fungi may have cutaneous manifestations, and so aseptic technique should be used to protect the laboratory workers.

2-2. **Specimen Processing.** Skin and nail scrapings are used primarily to diagnose superficial and cutaneous mycotic infections. The potassium hydroxide wet mount (described in detail in the next section of this chapter) is used in the examination of these specimens. In essence, we transfer a small fragment of the material into a drop or two of 10 percent potassium hydroxide (KOH) or sodium hydroxide (NaOH) on a clean glass slide and coverslip it for microscopic viewing. Other fragments may be mounted in lactophenol-cotton blue and similarly examined. Whether or not fungal elements are observed, fragments of the material should be inoculated routinely on two tubes of Sabouraud's dextrose agar—one to be incubated at 25° C. and the other at 37° C. When growth develops, macroscopic and microscopic morphological studies are conducted to establish identity.

2-3. Two superficial mycotic agents and certain of the cutaneous agents may be isolated from *hair*. It is best to examine the suspect area of the patient's scalp under a Wood's lamp (ultraviolet light) in a dark room to detect hairs that fluoresce with a bright, yellow-green color. Pluck some of the fluorescing hairs and place them in a sterile petri dish until time for examination. Remember, however, that tinea capitis, caused by most species of *Trichophyton*, and many infections with *M. gypseum* do not show fluorescence. If no fluorescent hair is seen, re-examine the patient under ordinary light and pluck hair stubs from the edge of the patches of infection. Hairs are cultured in the same manner as skin and nail scrapings. If infec-

TABLE 5
ROUTINE CULTURE PROCEDURE FOR SPUTUM

Culture Procedure	*Incubation Temperature*	
	25° C.	37° C.
Sabouraud's dextrose agar	2 slants	2 slants
Sabouraud's dextrose agar (ch and cy)[1]	2 slants	None
Brain heart infusion agar (ch and cy)	2 slants[2]	None
Brain heart infusion agar (with or without blood)	None	2 slants[3]

---

[1] Recommended antibiotics: chloramphenicol (to inhibit bacteria); cycloheximide (to inhibit saprophytic fungi).

[2] Some systemic fungi (*Histoplasma capsulatum*) exhibit poor growth on Sabouraud's dextrose agar at 25° C.

[3] The yeast form of dimorphic fungi may be inhibited at 37° C. if the antibiotics are present.

tions with *Piedraia hortai* or *Trichosporon cutaneum* are supected, Sabouraud's dextrose agar *without* chloramphenicol and cycloheximide should also be inoculated. Both fungi are sensitive to these antibiotics. (See table 4.)

2-4. Although pathogenic fungi isolated from *sputum* are generally systemic, we should recall that repeated isolation of saprophytes, such as *Aspergillus* or *Mucor* species, may be of clinical significance. A first-morning, 24-hour, or 3-day collection of sputum in a clean, sterile, widemouth, screw-capped bottle can be used. Centrifuge the sputum and examine the sediment directly on a clean glass slide under a coverslip. First scan under low power and then high power, using reduced illumination. Because more than one mycotic agent may give the same clinical signs, several routine identifying steps should be followed. After direct examination, use a Gram stain to demonstrate hyphae or blastospores. Fungal structures stain Gram-positive. An acid-fast stain will demonstrate the mycelium of *Nocardia* species. The Giemsa or Wright stain is useful for *Histoplasma capsulatum.* An India ink mount will reveal the encapsulated budding or nonbudding blastospores of *Cryptococcus neoformans.* Regardless of the presence or absence of fungal structures microscopically, the sediment should be cultured under the guidelines set forth in table 5.

2-5. The culture tubes should be checked daily for growth. Subculture them if contaminants threaten to overgrow the inoculated area. No culture tube should be discarded or reported as negative for at least 4 to 5 weeks. Growth at either 25° C. or 37° C., or both, and macroscopic morphology will provide tentative identification. Further identification may be accomplished by specific procedures to be covered in later chapters.

2-6. *Exudates, body fluids,* and *body tissues* require special mention. Collect exudates and pus from the active margin of open abscesses or ulcers. Material from closed lesions aspirated with a syringe by a physician should be inoculated directly to appropriate media or placed in sterile, screw-capped vials or tubes for later inoculation. Spinal fluid should be placed in sterile, screw-capped tubes. Other body fluids and tissue, such as peripheral blood, urine, synovial fluid, bone marrow, or biopsy material collected under aseptic conditions, may be inoculated directly to culture media. Large volumes that require centrifugation to concentrate them, or specimens that cannot be immediately processed can be temporarily stored in sterile, screw-capped tubes or bottles.

2-7. The culture techniques used for exudates, body fluids, and tissues are essentially the same as those for sputum. Note in table 6, however, that the etiological agent of rhinosporediosis, *Rhinosporidium seeberri,* cannot be cultured. The clinical material in suspected cases must be examined directly or fixed for histological examination.

2-8. **Preparation of Specimens for Shipment.** For a number of reasons, it is sometimes necessary to send a clinical specimen, or an unidentified fungus isolate, to a reference laboratory for further study. Breakage and possible contamination of the container can be avoided by proper attention to packaging. In forwarding cultures, 18- x 150-mm. tubes should be used. The growth of many fungi is inhibited during prolonged incubation in screw-capped tubes; therefore, send these specimens by airmail or by special messenger. Test tubes must always be wrapped individually in cotton or paper before placing them in a metal specimen container. Any free space in the specimen container is then stuffed with cotton or other packing material to prevent breakage. The specimen container is capped and placed in a cardboard mailing carton.

2-9. Fluid specimens obtained under *aseptic conditions,* i.e., blood, spinal fluid, or aspirated pus, may be shipped if they are tightly sealed in sterile vials or tubes and carefully packed. Hair, skin, and nail scrapings, or pure cultures of suspected pathogens may also be shipped, but urine, bronchial washings, or biopsied tissue usually become quickly overgrown with contaminants in transit, making the isolation of pathogenic fungi improbable. Cotton swabs should never be mailed because they will dry out. Of course, petri dishes are subject to breakage and leakage and, thus, should not be used for mailing purposes.

2-10. *Labeling information* needed by the reference laboratory includes:

- The patient's name, age, and sex.

Table 6

Specimens and Mexia for Demonstration and Isolation of Fungi from Specific Types of Fungus Diseases

Disease	*Type of Specimen*	*Isolation Medium*
Superficial Mycoses		
Piedra	Clipped hair	Sabouraud dextrose agar
Tinea nigra	Skin scrapings	Cycloheximide medium[1]
Tinea versicolor	Skin scrapings	Non available at present
Cutaneous Mycoses		
Candidiasis	Skin scrapings Scrapings from mucocutaneous areas Vaginal scrapings	Cycloheximide medium Sabouraud dextrose agar (for *Candida spp.* inhibited by cycloheximide) -
Onychomycosis	Nail scrapings	Cycloheximide medium
Tinea capitis	Plucked hair	
Tinea corporis	Skin scrapings	
Tinea pedis	Skin scrapings	
Subcutaneous Mycoses		
Chromoblastomycosis	Crust and scrapings from warty outgrowths Exudate from lesions	Cycloheximide medium
Mycetoma	Pus from draining sinuses Aspirated fluids from unopened sinus tracts Biopsy specimens	Sabouraud dextrose agar BHI[2] agar BHI (shake tube or anaerobic plates) for *A. israelii*
Rhinosporidiosis	Biopsied nasal and ocular polyps Skin scrapings	None available at present
Sporotrichosis	Pus from ulcers Aspirated fluid from subcutaneous abscesses	Cycloheximide medium
Systemic Mycoses		
Actinomycosis	Pus from draining sinuses Aspirated fluid from subcutaneous abscesses Sputum Spinal fluid Bronchial washings	BHI plates (anaerobic) BHI (shake tubes)
Blastomycosis, North American	Scrapings from edge of skin lesions	Cycloheximide medium
	Pus from open abscesses	BHI agar + C & S[3]
	Pus from sinus tracts Urine Sputum Bronchial washings	BHI (no antibiotics for 37° C. incubation
Blastomycosis, South American	Scrapings from edge of skin lesions Scrapings from mucous membranes Biopsied lymph nodes Sputum Bronchial washings	Same as for North American Blastomycosis
Candidiasis	Sputum Bronchial washings Spinal fluid Urine Stools	Cycloheximide medium Sabouraud dextrose agar (for *Candida spp.* inhibited by Cycloheximide)

TABLE 6 (Cont'd)

Disease	*Type of Specimen*	*Isolation Medium*
Coccidioidomycosis	Sputum Bronchial washings Spinal fluid Urine Scrapings from skin lesions Pus from draining abscesses and sinuses	Cychloheximide medium
Cryptococcosis	Spinal fluid Sputum Pus from abcesses Pus from sinus tracts Scrapings from skin lesions Urine	Sabouraud dextrose agar + chloramphenicol
Geotrichosis	Sputum Bronchial washings Stools	Sabouraud dextrose agar + chloramphenicol
Histoplasmosis	Blood Sternal marrow Sputum Bronchial washings Spinal fluid Pus from sinus tracts Exudate from ulcers Scrapings from skin lesions	Cycloheximide medium BHI agar + C & S BHI (no antibiotics) for 37° C. incubation
Nocardiosis	Sputum Spinal fluid Pus from abscesses Bronchial washings	Sabouraud dextrose agar (Incubate at both room temperature and 37° C.)
Miscellaneous Mycoses		
Aspergillosis	Sputum Bronchial washings	Sabouraud dextrose agar + chloramphenicol
Mucormycosis	Sputum Bronchial washings Biopsy material	Same as for Aspergillosis

---

[1] Sabouraud dextrose agar + chloramphenicol and cycloheximide.
[2] Brain Heart Infusion.
[3] Chloramphenicol and cycloheximide.

- Identification number or registration number.
- Name of the requesting physician and the submitting facility.
- Specimen origin and date of collection.
- Provisional diagnosis.

When cultures are submitted, such information as type of medium, date of inoculation, and incubation temperature are extremely helpful. Additional data which can be of value, when relevant, are the patient's record of residency and travel in the United States and foreign countries, and the results of any skin or serological tests.

3. Mycology Techniques

3-1. The techniques used in medical mycology are similar, in many respects, to those used in medical bacteriology. That is to say, we attempt to plant an organism on artificial culture media and grow it out in isolated colony form. The pure culture can then be studied from the standpoint of its morphology, physiological properties, and immunologic characteristics. With the mycotic agents, however, much greater emphasis is placed on differences in colony appearance and details of cell structure and arrangement. Correspondingly, less reliance is placed on fermentation reactions and serological tests. In this section, therefore, we will mention some of the techniques designed to make morphological studies of the fungi easier.

3-2. **General Considerations.** The mycologist uses a stiff, nichrome wire (22-gauge) with the last 4 or 5 mm. bent at right angles to the main shaft. The wire is fixed firmly in a metal handle for ease in transferring mycelial growth. A pair of short, stiff teasing needles are useful in pulling apart dense mycelium for microscopic examination. Test tubes of fairly large diameter (18- x 150-mm.) afford a larger butt, more resistant to drying; hence, these tubes are preferable because of the longer period often necessary for adequate fungal growth. Tubes should have cotton plugs in preference to screw caps because plugs permit oxygen to enter and also allow the slant surface to remain dry through air exchange with the outside. A dry surface gives better sporulation and better pigment production.

3-3. Transfer of cultures should be done over absorbent paper (paper toweling) moistened with a fungicidal agent. It is also wise to use a small vial of sand moistened with a fungicidal agent to remove excessive fungal material from the wire before you flame it. This move prevents dangerous spattering of infectious material as the wire is sterilized in the flame.

3-4. **Wet Mounts.** The technique of wet mounting is used for preparing specimens either from patients or cultures for microscopic viewing. The potassium hydroxide (KOH) wet mount is perhaps the most widely used of these. Let's assume a patient with a lesion on his arm has been sent to the mycology laboratory. We wash the lesion area thoroughly with 70 percent alcohol to remove any dirt particles, bacteria, and medication which might interfere with microscopic examination. The outer, active margin of the lesion may then be scraped with a sterile scalpel blade. The scrapings are deposited directly into a sterile petri dish. We transfer a small fragment of the material into a drop or two of 10 percent KOH on a clean glass slide and coverslip it. Let the preparation sit at room temperature for 15 to 30 minutes so that tissue and debris will dissolve in the KOH. Then examine the slide under low, then high magnification. To save time, the slide may be heated gently for a few seconds over a flame. *Do not boil* the KOH. The strong alkali serves as a clearing agent, making the fungal elements (spores and/or mycelium) more easily discernible. The KOH mount informs us only of the presence or absence of fungal elements in the tissue scrapings. A positive identification requires culturing and additional observation of the ensuing growth.

3-5. Lactophenol-cotton blue wet mounting fluid is the basic stain used in mycology. It serves a threefold purpose. The lactic acid in it acts as a preservative; phenol serves as a fungicidal or killing agent; and cotton blue (Poirrier's blue) lends color to the structures and makes them more readily observable.

3-6. Ordinarily, the specimen used for making *teased, wet mount* preparations is obtained from a fungus growing on the surface of a culture medium. A small quantity of the mycelium is removed with a stiff needle and scraped off into 1 or 2 drops of lactophenol-cotton blue mounting fluid placed on a clean glass slide. Next, *gently* pull the mycelium apart so that individual structures will be clearly visible. Rough handling will destroy the all-important spore-hyphal relationship, or natural arrangement, and make identification difficult. The slide may now be coverslipped and examined under the microscope. The slide can be preserved for later use by sealing the edges with fingernail polish or varnish.

3-7. Some workers prefer the *scotch tape wet mount* to the teased preparation because the scotch tape technique is relatively simple, it subjects the fungus to considerably less trauma, and it preserves the structural relationship that is essential for accurate identification. There are two disadvantages to this technique: Visual acuity is partially lost due to the tape itself, regardless of its clarity; and tape does not lend itself to the preservation of slides for future study because there is a tendency for the tape to loosen. A piece of clear scotch tape (not magic tape) approximately 1 sq. cm. in size is used. We unplug the tube containing the fungal growth and touch the sticky surface of the scotch tape to the mycelium with forceps. The

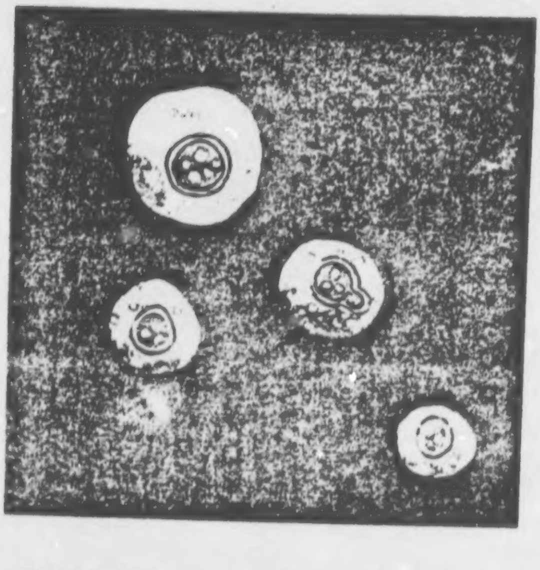


Figure 8. Cryptococcus neoformans in India ink wet mount.

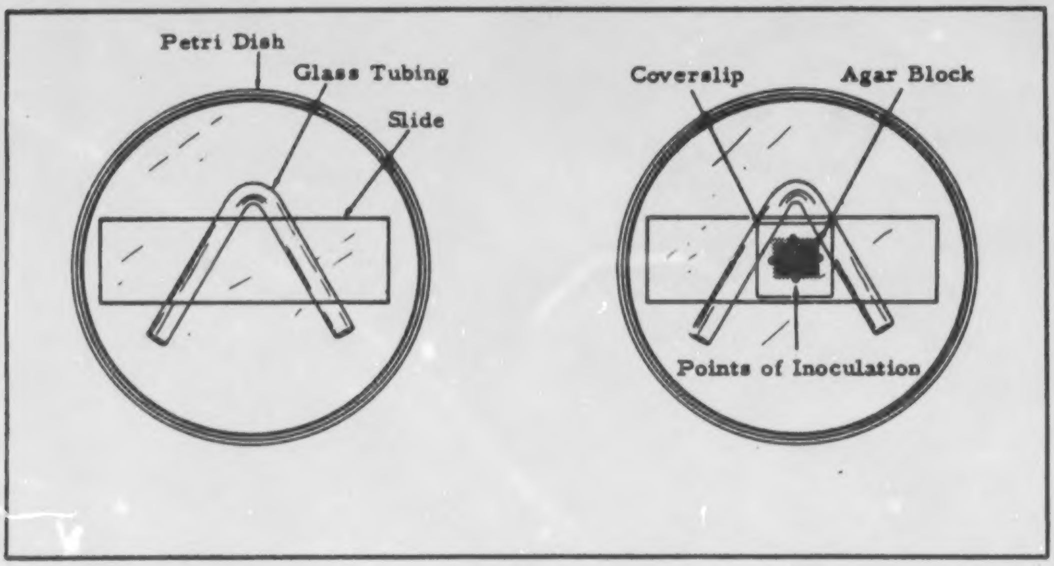


Figure 9. Procedure for slide cultures.

tape is then placed (sticky surface down) on top of 1 or 2 drops of lactophenol-cotton blue on a clean glass slide. Press the tape gently but firmly against the glass slide. The mount may then be examined microscopically.

3-8. The *India ink wet mount* technique is used primarily to detect *Cryptococcus neoformans* in spinal fluid, but other body fluids may be similarly examined. After we centrifuge the specimen, we transfer a loopful of the sediment to a clean glass slide and mix the material with a small drop of undiluted India ink. After coverslipping, we examine the slide microscopically. Since the ink is unable to penetrate the large capsule surrounding *Cryptococcus neoformans*, the capsule appears as a clear disc against a black background. We also see a centrally-located dense, single or budding cell within the disk. Figure 8 provides an example. The sole purpose of the India ink is to reveal the capsule of *C. neoformans* as an aid to accurate diagnosis. If the wire loop is used for mixing spinal fluid and ink, allow the wire to cool sufficiently to prevent precipitation of the ink by heat.

3-9. **Culture Methods.** There are three principal techniques used in cultivating the fungi: the routine tube or bottle culture, the slide culture, and cut-streak inoculation of plated media. Normally, the *routine tube* or *bottle culture* is used to isolate fungi from clinical specimens. Although cotton-plugged tubes are recommended for the reasons we have previously mentioned, 4-ounce prescription bottles with screw caps are used successfully by some workers. Petri dishes are unacceptable for cultures since highly virulent spores are readily disseminated by air currents when the lid if removed. Cultures incubated at 37° C. tend to dry out rapidly, so large tubes (18- x 150-mm. or even 25- x 150-mm.) containing more media are preferred. Sabouraud's dextrose agar is the most widely accepted medium for initial isolation. It is prepared in slant form.

3-10. Chloramphenicol or cycloheximide, or both of these inhibitory substances, may be added to the medium as recommended in table 6 to retard growth of contaminants. The clinical material is inoculated with the 22-gauge nichrome needle by making two or three deep cuts into the medium at about the midpoint of the slant surface. At least two tubes should be inoculated in order to permit incubation at both 25° C. and 37° C. Be sure that all tubes are properly labeled prior to incubation.

3-11. Gross examination of inoculated tubes should be carried out on a routine daily basis. The following growth characteristics aid appreciably in the final identification of the fungus: rate of growth, colony size, and surface topography (flat, heaped, folded, or smooth); texture (yeastlike, glabrous, powdery, granular, velvety, or cottony); and surface pigmentation on both front and reverse sides of the slant.

3-12. Using either the teased or scotch tape wet mount, we can now examine the colony microscopically. It may be possible to identify the fungus by this direct examination if spores or other diagnostic structures are present. Otherwise, additional procedures may be necessary, i.e., slide cultures for maintaining structural relationships, and spe-

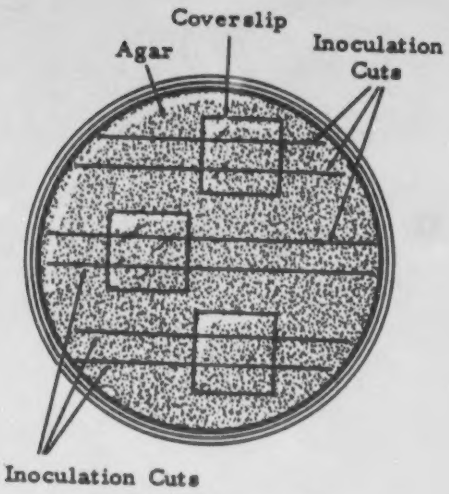


Figure 10. Procedure for coverslip over cut of Candida albicans.

cial media conductive to sporulation or production of other diagnostic structures. If facilities are available, physiological testing and animal inoculation may be used to help arrive at final identification of the mycotic agent.

3-13. If a teased or scotch tape mount fails to yield sufficient information for identification, or in the event that permanent study slides are desired, the *slide culture* for *monomorphic molds* can be set up. This procedure, shown in figure 9, uses a petri dish containing a bent glass rod, a coverslip, and an ordinary microscopic slide, all of which have been wrapped in metal foil and sterilized. With a sterile scalpel blade, cut approximately 1 square centimeter of medium from a plate of Sabouraud's dextrose agar or potato dextrose agar. Place it aseptically on the center of the microscopic slide atop the bent glass rod within the petri dish. Inoculate each of the four sides of the square of medium by making a cut of about 1 millimeter into the medium. Cover the square of inoculated medium with the coverslip and add 8 to 10 drops of sterile water to the bottom of the petri dish. Replace the top of the petri dish and incubate the culture at 25° C. until growth appears.

3-14. The slide culture may be examined microscopically without disturbing the coverslip. When the desired stage of growth has been reached, a lactophenol-cotton blue preparation may be made by gently lifting the coverslip (with its adhering fungus) and laying it on a microscopic slide holding 1 or 2 drops of the dye. Permanent mounts can be made by blotting the excess dye around the edges of the coverslip and sealing the coverslip with fingernail polish or varnish. Highly virulent molds should not be grown in slide culture because of the danger of infection to persons handling them.

3-15. The *cut streak inoculation* for yeastlike fungi is used for microscopic observation of undisturbed yeast structures, especially the chlamydospores of *Candida albicans*. It is essential that a medium conducive to chlamydospore production be used. Among these media are corn-meal agar with 1 percent tween 80, and commerical chlamydospore agar. With a heavy inoculating needle, some of the suspect material is inserted into the medium by cutting to the bottom of the plate. Make the cut at an angle of 45° to allow greater area for growth beneath the agar surface. The cut must extend across the plate.

3-16. Place a sterile coverslip over the cut, as we have shown in figure 10. As many as four or five specimens may be cut into the same plate to conserve agar, and one of these inocula should be a positive control consisting of a known culture of *C. albicans*. The dish is incubated at 25° C. or room temperature for at least 2 days. Some yeasts produce fruiting structures more plentifully under microaerophilic conditions, as under the coverslip and down in the medium. Others are found away from the coverslip on the surface of the plate, e.g., arthrospores, blastospores, hyphae, and pseudohyphae. Unless sufficient growth adheres to the coverslip, the preparation of permanent mounts by the cut-streak technique is not possible.

3-17. **Safety Precautions.** Ideally, every laboratory performing diagnostic work in mycology should be equipped with a bacteriological safety hood operating under negative air pressure to draw fungus spores away from the technician, should they become airborne. Since possession of a hood is probably the exception rather than the rule, it serves to emphasize the extreme importance of exercising the most careful aseptic technique. All the precautions taken in bacteriological work are equally applicable to mycology. Remember, too, what we stated earlier about transferring cultures over a disinfectant-soaked towel, and how to avoid spattering when you flame the wire needle. Probably a good rule-of-thumb is to handle each specimen as if it contained a highly virulent mycotic agent. In the event of an accident, such as splashing of infectious material into the eye or on open skin, report this immediately to a physician. If you drop a culture, cover the debris with disinfectant. As with any other type of accident in microbiology, use good judgment and be prepared to institute decontamination measures immediately.

The Yeastlike Fungi and the Monomorphic Molds

ALTHOUGH THE yeastlike fungi have many morphological and physiological characteristics identical to the true yeasts, they differ in that the former lack the ability to reproduce sexually. All the fungi in this group produce moist colonies with a creamy consistency, at least in their early growth phase. These organisms are fundamentally unicellular in nature, however, some members produce both a pseudomycelium and a true mycelium. The medically important yeastlike fungi will be discussed in the opening section. The remainder of the chapter will consider the monomorphic molds.

2. The monomorphic molds multiply only in filamentous form, regardless of whether their environmental temperature is 25° C. or 37° C. Growth is characterized by tubular branching septate hyphae which intermingle to form a mass of mycelium on artificial media as well as natural substrate. The aerial hyphae develop modified branches, called phores, upon which a diversity of conidia are proliferated. Generally, mold growth is dry and dusty, particularly after sporulation. The majority of fungi pathogenic for man are monomorphic molds.

3. We will cover the clinical aspects and laboratory diagnosis of the monomorphic molds commencing with the least severe superficial forms. The dermatophytes (cutaneous monomorphic molds) and the subcutaneous agents will be discussed from the same standpoint. A section regarding the deep-seated diseases caused by the systemic monomorphic molds will complete this chapter.

4. The Pathogenic Yeastlike Fungi

4-1. Pathogenic species of the yeastlike fungi are frequently recovered from clinical specimens, such as skin scrapings, sputum, mucocutaneous swabs, and feces obtained from individuals who demonstrate no adverse symptomatology. This means that the organisms are present in or on the host but do not become invasive or toxic unless certain physiological changes occur either locally or systemically to alter susceptibility to infection. Some of the conditions permitting these opportunistic organisms to cause disease include traumatic injury, chronic debilitating and metabolic diseases, and the increased use of steroids and antibiotics. In order to firmly establish the taxonomic relationship of the yeastlike fungi, you are referred to table 7.

4-2. **Isolation and Identification.** As in all phases of medical mycology, the clinical material is examined directly in wet preparation for the presence of large, unicellular budding cells and pseudo or true mycelium. *Candida species* reproduce primarily by means of simple budding, but they have the ability to form pseudo and true mycelium. "Pseudomycelium" is the term applied when the daughter cell fails to detach from the mother cell. If this process is repeated a chain of daughter cells may result, forming a fragile filament closely resembling true mycelium. True mycelium is formed by septation of a germ tube developed from a mother cell. Quite similar to the true yeasts are the members of the Genus *Cryptococcus*. All members of this genus have the ability to encapsulate, and reproduction is solely by simple budding. *Geotrichum spp.* never reproduce by simple budding and therefore are the most atypical of the yeastlike group. Instead, true mycelium which readily breaks up into rectangular arthrospores is formed. The Genus *Trichosporon* reproduces by means of blastospores, and the formation of a true mycelium which fragments into arthrospores. Members of the Genus *Pityrosporum* are unicellular, reproducing only by means of simple budding. The buds are separated from the mother cell by a cross-wall, which makes identification relatively simple. If any of these yeastlike structures are seen in direct preparation of the specimen, cultural studies must be instituted.

4-3. *Cultivation.* It is essential that organisms be isolated in pure form in order for you to perform accurate morphologicol procedures and other tests that may be necessary. Probably the best

TABLE 7

CLASSIFICATION OF THE YEAST LIKE FUNGI

CLASS: DEUTEROMYCETES (FUNGI IMPERFECTI)
    ORDER: PSEUDOSACCAROMYCETALES
        FAMILY: CRYPTOCOCCACEAE
            Sub-Family I - Cryptococcoidiae
                A. Genus - *Cryptococcus*
                B. Genus - *Pityrosporum*
            Sub-Family II - Candidoideae
                A. Genus - *Candida*
                B. Genus - *Trichosporon*
                C. Genus - *Geotrichum*

method of separating mixed yeast populations is to inoculate the specimen into a tube of Sabouraud's dextrose broth. This broth is identical to Sabouraud's dextrose agar, except it contains no agar and will not solidify. It is available in dehydrated form. After overnight incubation at 37° C., the tube is gently shaken and a loopful streaked to a blood agar plate. Following 24 or more hours of incubation, colonies of different types are picked and inoculated on slants of Sabouraud's dextrose agar for incubation at 37° C. The growth from individual colonies should now be in pure form on the slants. In order to prevent bacterial growth, a 0.05-mg./ml. concentration of chloramphenicol should be incorporated into the medium, while the addition of cycloheximide in a concentration of 0.5 mg./ml. will prevent the growth of saprophytic fungi. Dehydrated media containing the above constituents in proper concentration are available. If bacterial overgrowth still results, four tubes of Sabouraud's dextrose broth to which 1 drop, 2 drops, 3 drops, and 4 drops of N HCL have been added, respectively, are inoculated with the original isolate. After overnight incubation at 37° C., a loopful of broth is transferred from the acid tube showing no bacterial growth to a blood agar plate. Following overnight incubation at 37° C., colonies are picked for inoculation to Sabouraud's dextrose agar.

4-4. *Examination of pure cultures.* Pure cultures of the yeastlike fungi must be studied microscopically to determine what structures are present. The absence of mycelium is indicative of a species of *Candida, Cryptococcus*, or a true yeast. The presence of mycelium with both arthrospores and blastospores is strongly suggestive of the Genus *Trichosporon.* When only arthrospores are formed the unknown is most likely *Geotrichum candidum.* When the growth is very slimy and mucoid, and only encapsulated blastospores are seen microscopically, it is likely to be a *Cryptococcus spp.* A definitive identification, however, is based on a combination of physiological and biological tests which will be discussed later in this section. If *Candida albicans* is suspected as a result of finding blastospores, pseudo and true mycelium in wet preparation, the growth should be inoculated to chlamydospore or corn meal agar plus 1 percent tween 80 using the cut streak technique described in Chapter 1.

4-5. **Candida Albicans.** Candidiasis, formerly known as Moniliasis, is the disease caused by *Candida albicans.* This organism has no geographic limitation, however, in regions of the world where carbohydrates make up a large portion of the diet and profuse sweating is commonplace, the incidence of infection is greater than elsewhere. The fungus is a normal inhabitant of the intestinal tract, and its incidence of that region of the body has been shown to increase with age. Since *Candida albicans* can be found at some point in or upon almost all humans, Candidiasis infections are considered endogenous in origin. The organism is usually classified as a cutaneous fungus, and its occurrence in systemic diseases was for many years a rarity. The therapeutic use of steroids and antibiotics over extended periods has resulted in a significant increase in systemic infections.

4-6. *Candida albicans* has a particular affinity for the mucous membranes of the oral cavity where it produces confluent or discrete patches that bleed quite readily. This form of the disease is called thrush. It is found in newborn infants who probably were infected during passage through the

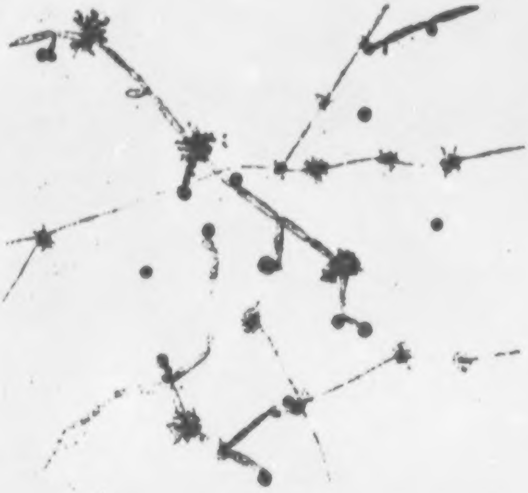


Figure 11. Candida albicans.

TABLE 8
DIFFERENTIATION OF SPECIES

Species	Morphology on Corn Meal Agar + 1% tween 80 or Chlamydospore Agar Cut-streak plate	Sabouraud dextrose broth Growth characteristics	Sugar* fermentation reactions				Sugar Assimilation					
			D	M	S	L	Dextrose	Galactose	Lactose	Maltose	Raffinose	Sucrose
C. albicans	Irregular or spherical clusters of blastospores of septa. Chlamydospores single or in clusters. May be very numerous (chlamydospores do not develop at 37° C.)	No surface growth	Ag	Ag	A or O	O	+	+	O	+	O	+
C. guilliermondii	Very fine mycelium. Small clusters of blastospores at the septa.	No surface growth	O or Ag	O	O or Ag	O	+	+	O	+	+	+
C. krusei	Elongate cells forming a branched mycelium easily disintegrated. "Crossed sticks" at septa.	Wide surface film	Ag	O	O	O	+	O	O	O	O	O
C. parapsilosis	Fine and course mycelium (giant forms). Blastospores single or in short chains at septa or distal ends of cells.	No surface growth	Ag or A	O or A	O or A	O	+	+	O	+	O	+
C. pseudotropicalis	Very elongate cells which readily fall apart and lie parallel. "Logs in stream".	No surface growth	Ag	O	Ag	Ag	+	+	+	O	+	+
C. stellatoidea (probably a variant of C. albicans)	More extensive mycelium with irregular or spherical clusters of blastospores at septa. Chlamydospores rare (may show a supporting cell).	No surface growth	Ag	Ag	A or O	O	+	+	O	+	O	O
C. tropicalis	Blastospores anywhere along mycelium or in irregular clusters. Chlamydospores very rare.	Narrow surface film with bubbles	Ag	Ag	Ag	O	+	+	O	+	O	+

---

* D = dextrose M = maltose S = sucrose L = lactose

vagina where *Candida albicans* exists, generally asymptomatically, in a large number of women. Thrush is also common in diabetics, people on prolonged corticosteroid or antibiotic therapy, and the aged.

4-7. Another form of the disease, vulvovaginal candidiasis, occurs sometimes in diabetic and pregnant women, probably due to the higher sugar content of the urine or vaginal secretions. The disease may develop wherever two skin surfaces are in contact over such long periods that perspiration cannot evaporate. The breakdown of urea in the perspiration to ammonia results in chemical irritation and allows the organism to become invasive. *Candida albicans* is a common invader of the nails and toes. It causes a wet, weeping variety of tinea pedis (athlete's foot).

4-8. Pulmonary candidiasis is sometimes diagnosed in conjunction with some other respiratory disease. This is considered a secondary invasion by the fungus. Endocardial candidiasis, encountered in drug addicts who fail to sterilize their needles and syringes, is a disease similar to subacute bacterial endocarditis. One last form of infection, disseminated candidiasis, involves the vital body organs. It is generally associated with the terminal stages of some other fatal disease.

4-9. Laboratory identification of *Candida albicans* first involves the direct microscopic examination of clinical material, such as skin and nail scrapings in a potassium hydroxide wet mount. Mucosal scrapings are examined either on a Gram-stained smear or in wet mount using lactophenol cotton blue. The sediment of specimens such as spinal fluid, bronchial washings, and sputum are examined similarly. If the typical picture of budding yeast cells and mycelium pinched in at the septations is seen, a preliminarily report of "Forms suggestive of *Candida*" is indicated. In such cases it is helpful to the physician to indicate the number of organisms per microscopic field. See figure 11.

4-10. The young colonies on Sabouraud's dextrose agar at 37° C. are white, soft, and generally have smooth surfaces and borders. Older colonies frequently show a fringe of submerged mycelium appearing as feathery outgrowths deep in the agar. See detail B of foldout 1 for the macroscopic morphology of *C. albicans* on Sabouraud's dextrose agar. If microscopic examination of wet mounts reveals structures suggestive of *Candida*, it is then essential to establish whether or not the culture is *C. albicans* or a saprophyte.

4-11. There are several procedures which may be used to confirm identification of *C. albicans*. Probably the most common is the demonstration of chlamydospores, using the cut streak technique described in Chapter 1. If the cut streak procedure fails to demonstrate chlamydospores, sugar fermentation and assimilation tests shown in table 8 should be accomplished for positive identification.

4-12. Another, more rapid method of identifying *C. albicans* is based on the fact that this fungus, alone, will form germ tubes on Levine's eosin-methylene blue medium when incubated at 37° C. in a candle jar. The medium should be streaked with the suspect organism as well as a known positive control of *C. albicans*. The jar may be opened after 4 to 6 hours of incubation and the colonies examined for the formation of germ tubes by the yeast cells of *Candida albicans*. If there is no germ tube formation evident in the unknown, the culture may be reincubated and examined again after 12 hours.

4-13. Serological and animal pathogenicity tests are of no value in the laboratory diagnosis of *Candida albicans*. *Candida spp.* other than *albicans* are rarely agents of disease and their identification is usually not necessary in diagnostic work. One exception occurs in cases where the organism is repeatedly isolated from clinical material that is normally sterile, or when no other recognized pathogenic organism has been recovered from the lesion or diseased clinical material.

4-14. **Cryptococcus Neoformans.** Cryptococcosis formerly known as torulosis, European blastomycosis, or Busse-Buschkes disease, is worldwise in distribution in both animals and man. The causative agent, *Cryptococcus neoformans*, has a particular affinity for the central nervous system; however, skin, bones, and other organs may also be infected. Its prevalence is probably underestimated as a result of failure to suspect its presence. *C. neoformans* has been recovered from areas in or upon the bodies of many individuals who indicate no adverse symptomology. In the 1950s it was discovered that *C. neoformans* is saprophytically associated with pigeon droppings found under roosting sites.

4-15. Inhalation of spores borne on dust particles (causing pulmonary cryptococcosis) is by far the most common portal of entry. *C. neoformans* has also been cultured from the blood of infected patients. Some workers are of the opinion that meningitis may result directly from a nasopharyngeal involvement. At the present time, proof is lacking that a primary cutaneous form of the disease occurs. Primary pulmonary cryptococcosis gives rise to chronic lung infections. Although the pulmonary disease may prove fatal, dissemination to the central nervous system usually occurs first. It is likely that many pulmonary cryptococcosis infections go undetected and heal spontaneously, as is the case with some of the other deep mycoses. Disseminated cryptococcosis generally in-

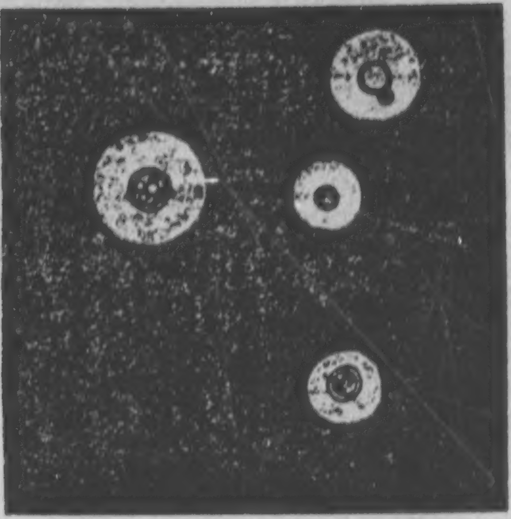


Figure 12. Cryptococcus neoformans.

volves the central nervous system. The spinal fluid is discolored a dirty yellow; the protein and cell count elevated (predominately lymphocytes), and the sugar is reduced.

4-16. Direct examination of clinical materials such as sputum, urine, or spinal fluid is accomplished with the India ink wet mount. (See fig. 12.) If the volume of fluid permits, these specimens should be centrifuged and the sediment used in making the preparation.

4-17. Regardless of the microscopic findings, the clinical material should be cultured on Sabouraud's dextrose agar without cycloheximide, as *C. neoformans* is sensitive to this antibiotic. Inoculation of an enriched medium such as blood or brain heart infusion agar is also recommended. Usually, the saprophytic cryptococci are unable to grow at 37° C. It should not be assumed, on the other hand, that any *Cryptococcus* which grows at 37° C. is *C. neoformans* because certain saprophytic strains do not conform to this pattern. The colonial morphology may take 10 to 14 days to develop to a stage where macroscopic evaluation is possible. The colonies are white or cream to tan, and soft and creamy in texture. See foldout 1, detail C. If capsular material is produced, the colonies are slimy or mucilaginous.

4-18. When growth is adequate there are other tests useful in identifying *C. neoformans*. Only members of the *Genus Cryptococcus* have the ability to produce urease, and on this basis the cryptococci may be separated from the true yeasts and other yeastlike fungi. In order to determine whether the *Cryptococcus spp.* is *C. neoformans* or a saprophytic form requires further testing. *C. neoformans* differs from the saprophytic cryptococci in its pattern of nitrate and sugar assimilation seen in table 9. A pathogenicity test using mice inoculated intracerebrally may be used instead of the nitrate and sugar assimilation tests. The mice are sacrificed at the end of 2 weeks and the gelatinous brain material examined for encapsulated, budding yeast cells. Serological testing has proved of little or no value.

TABLE 9

DIFFERENTIATION OF CRYPTOCCOCCUS NEOFORMANS FROM CRYPTOCOCCUS SPP.

Test	*Cryptococcus neoformans (pathogen)*	*Cryptococcus sp. (saprophyte)*
Incubation at 25° C.	Slow to moderate rate of growth; smooth, moist, mucoid, cream colored to tan.	Slow to moderate rate of growth; smooth, pasty, and cream colored. (A *very few* species might look like *C. neoformans* at 25° C.)
Incubation at 37° C.	Better growth than at 25° C., characteristic shiny, mucoid, and glistening growth.	No growth or very little growth (some exception).
India Ink Wet Mount	Thick capsules present	Capsules present or absent, usually thin if present.
Urea Test (agar medium)	Positive The urea test rules out other yeasts (especially the *Saccharomyces*,) which are urea negative.	Positive
Nitrate Assimilation Test	Nitrate, KNO_3, *not* assimilated	Nitrate, KNO_3, assimilated
Animal Pathogenicity Test	Positive	Negative

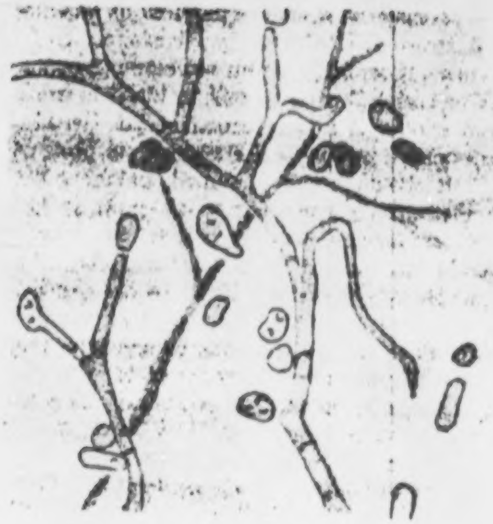


Figure 13. Geotrichum canididium.

4-19. **Geotrichum Candidum.** This organism is the causative agent of the disease—geotrichosis. Since *G. candidum* is commonly present in the gastrointestinal tract, usually as a saprophyte, a diagnosis is dependent on repeated demonstration of this fungus in direct examination of clinical material and the absence of any other pathogenic agent.

4-20. *G. candidum* has been implicated in mild respiratory infections. Coughing and the production of blood-streaked sputum may result. On rare occasion the fungus has been recovered in cases of colitis. *Geotrichum candidum* is also a frequent secondary invader, and therefore, its role in disease is difficult to assess.

4-21. Identification of *Geotrichum candidum* is based on its colonial and microscopic morphology. Direct mounts of clinical material demonstrate large (4 to 10 microns), round, oval, or rectangular cells which may or may not be joined together to form wide mycelial filaments. See figure 13 for the microscopic appearance of *Geotrichum candidum.*

4-22. *Geotrichum candidum* in its early growth phase appears similar to the other yeastlike organisms, both colonially and microscopically. (See FO 1, detail D.) As the colony matures, it takes on a fluffy mycelial character which is readily observable. Microscopically, this fluffy mycelial phase appears in the form of chains of rectangular arthrospores; there may also be many individual disarticulated, cylindrical, barrel-shaped, or sometimes eliptical arthrospores.

4-23. **Trichosporon Cutaneum.** White piedra, is a relatively rare superficial mycosis of man which affects the hair of the scalp or body. The agent responsible for the disease, *Trichosporon cutaneum* (formerly known as *Trichosporon bergelii)* not only grows on the hair, but also invades the hair shaft, causing it to disintegrate and break off.

4-24. Hairs suspected of being infected with *T. cutaneum* are plucked and examined in 10-percent KOH wet mount. If the organism is present, you will see soft nodules, ranging from white to light brown in color, loosely attached to the hair shaft. Arthrospores and blastospores may also be observed.

4-25. Further confirmation of identify will require culturing on Sabouraud's dextrose agar with cycloheximide. The typical colony is soft in consistency and cream to white in color, later becoming greyish, wrinkled and heaped. (See FO 1, detail E.) Microscopic morphology is characterized by a true mycelium which produces both blastospores and arthrospores. Biochemically, the fungus shows an inability to ferment sugars or to assimilate glucose, galactose, sucrose, maltose, and lactose. Typical microscopic morphology may require use of the slide culture described in Chapter 1.

4-26. **Pityrosporum Ovale.** The members of the Genus *Pityrosporum* are common saprophytes on the skin of man. *Pityrosporum ovale* was once erroneously considered to be a causative agent of dandruff. The organism requires oleic acid, which is found in the sebaceous material abundantly produced by the scalp. Identification is necessary to insure differentiation from the pathogenic yeastlike fungi. (See fig. 14.)

4-27. *Pityrosporum ovale* can be cultured on ordinary media if fatty substances containing oleic acid are added. The yeastlike colony ranges in color from cream to tan. Identification is based on the microscopic observation of very small, yeastlike cells. The bud is separated from the mother cell by a cross-wall. There is no mycelial formation.

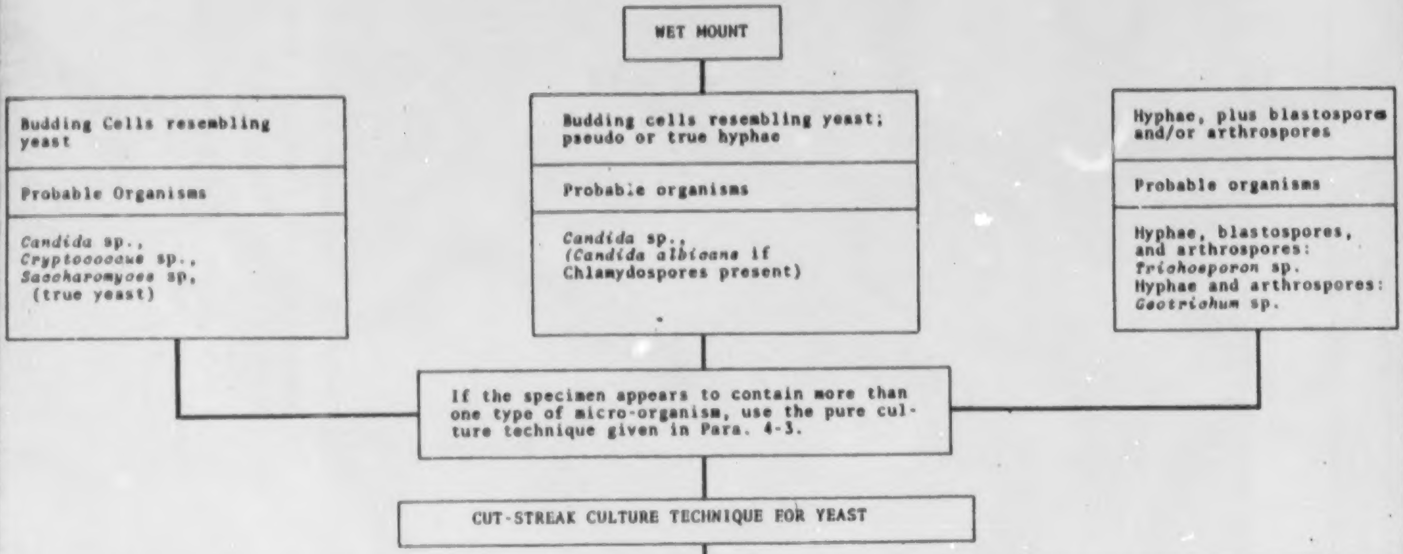
5. The Superficial Monomorphic Molds

5-1. There are three medically important mycotic agents in this category: *Piedraia hortai, Cladosporium werneckii,* and *Malassezia furfur,* as shown in table 10.

5-2. **Piedraia Hortai.** This is the causative mycotic agent of the hair infection, black piedra. Hard, brown or black nodules are formed along the shafts of the hair of the scalp. Other than a clicking sound when the hair is combed, the disease is asymptomatic. These modules are hard enough to cause a gritty sound when pressed between glass slides. Black piedra occurs primarily in Asia and Latin America.

5-3. Identification of *P. hortai* is based on microscopic examination of KOH mounts of suspect

LABORATORY IDENTIFICATION OF THE YEAST-LIKE FUNGI



WET MOUNT

Budding Cells resembling yeast

Probable Organisms

Candida sp.,
Cryptococcus sp.,
Saccharomyces sp,
(true yeast)

Budding cells resembling yeast; pseudo or true hyphae

Probable organisms

Candida sp.,
(*Candida albicans* if
Chlamydospores present)

Hyphae, plus blastospores and/or arthrospores

Probable organisms

Hyphae, blastospores, and arthrospores:
Trichosporon sp.
Hyphae and arthrospores:
Geotrichum sp.

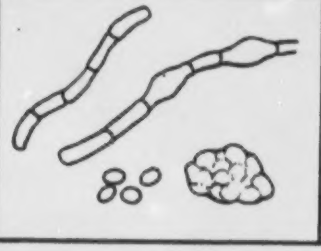
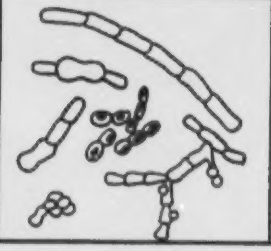
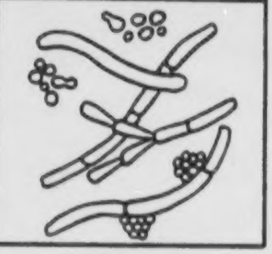
If the specimen appears to contain more than one type of micro-organism, use the pure culture technique given in Para. 4-3.

CUT-STREAK CULTURE TECHNIQUE FOR YEAST

POSSIBLE RESULTS FROM CUT-STREAK CULTURE TECHNIQUE FOR YEAST	ORGANISMS	CONFIRMATION
Hyphae and arthrospores	*Geotrichum Candidum*	morphology; exclusion of other organisms Para. 4-22.
Hyphae and blastospores	*Candida* sp.	Table 6 and Para. 4-11
Hyphae, arthrospores and blastospores	*Trichosporon* sp.	Para. 4-24 and 4-25
Hyphae, blastospores and chlamydospores	*Candida albicans*	Table 6, Para 4-11 and 4-12
No Hyphae, only blastospores	*Cryptococcus* sp. *Saccharomyces* sp.	Table 7 and Para. 4-17, 4-18
Small blastospores, cross walls between mother and daughter cell	*Pityrosporum* sp.	Microscopic Morphology Para. 4-27

Figure 14. Laboratory Identification of the yeastlike fungi.

TABLE 10
MORPHOLOGICAL CHARACTERISTICS OF SUPERFICIAL MONOMORPHIC MOLDS

Morphological Characteristics	*Piedraia hortai*	*Cladosporium*	*Malassezia furfur*
Growth rate	slow	slow	not cultured on artificial media
Colony texture	velvety	moist, smooth to velvety	not applicable
Topography	raised or flat with cerebriform undulations	flat	not applicable
Surface color	dark, greenish-black to black	shiny, dark greenish-black to black	not applicable
Back (reverse) color	same as surface	same as surface	not applicable
Microscopic	dark, thick-walled closely septate hyphae; chlamydospores, asci and ascospores may be present	dark, branched, septate hyphae (1.5-3M in D.); blastospores, chlamydospores, and 1-3 celled conidia may be present	branching, septate hyphae; grape-like clusters of thick-walled spores (3-8 M in D.) along the hyphae.
Sketch of microscopic appearance in wet mount			

hairs as well as colonial morphology. The fungus grows well on cycloheximide agar, producing black to greenish-black colonies which are smooth and raised. Microscopically, the colony from culture is found to consist of dark, closely septate filaments of hyphae, chlamydospores and on rare occasion asci. Microscopic examination of the hair nodule will reveal many oval asci containing 2 to 8 ascospores encased in a darkly pigmented mycelium.

5-4. **Cladosporium Werneckii.** This is the etiological agent of the asymptomatic disease called tinea nigra. The disease is characterized by the development of dark brown to black blotches on the palms of the hand, or on rare occasion, other parts of the body. Generally, only one lesion is present; however, there may be multiple areas of infection on one hand. Although considered a tropical disease, a few scattered cases have been reported in the U.S.

5-5. *Cladosporium werneckii* appears as dematiaceous (pigmented black or dark brown) branched hyphae when observed in 10-percent KOH mounts of suspect lesions. The organism grows readily but slowly on cycloheximide agar. It produces a moist, yeastlike, greenish-black raised colony. Older colonies develop a grey-black serial mycelium. Microscopic examination of early colonial growth reveals spherical or oval budding cells, many of which are divided centrally by a cross-wall. Short chains of these cells are common. Mounts from the periphery of older colonies reveal dematiaceous, septate hyphae along which clusters of blastospores develop.

5-6. **Malassezia Furfur.** The mycotic agent responsible for the disease, tinea versicolor, is *Malassezia furfur*. This superficial infection of the skin characteristically produces scaly brown patches in such areas as the chest, back, neck, axillae, face, arms, thighs or groin. These patches cause occasional itching; if exposed to the sun, they tan unevenly. This chronic disease is found worldwide; however, it is most prevalent in the tropics.

5-7. Although areas infected with *M. furfur* will fluoresce under ultraviolet light, identification rests on microscopic examination of skin scrapings in 10-percent potassium hydroxide or methylene blue. The use of transparent scotch tape has also been suggested. When the sticky surface of the tape is pulled from the lesion, it carries the scales loaded with the fungus, which may then be placed

TABLE 11
ECOLOGY OF DERMATOPHYTES

ANTHROPOPHILIC AGENTS

Microsporum audouinii
Microsporum distortum
Microsporum ferrugineum
Trichophyton concentricum
Trichophyton megninii
Trichophyton mentagrophytes (downy)
Trichophyton rubrum
Trichophyton schoenleinii
Trichophyton soudanense
Trichophyton tonsurans
Trichophyton violaceum
Trichophyton yaoundei

GEOPHILIC AGENTS

Keratinomyces ajelloi
Microsporum cookei [1]
Microsporum gypseum
Trichophyton terrestre [1]

ZOOPHILIC AGENTS

Microsporum canis
Microsporum vanbreuseghemii
Trichophyton equinum
Trichophyton gallinae
Trichophyton mentagrophytes (granular)
Trichophyton verrucosum

[1] Saprophytic for humans.

on a microscopic slide, with or without stain and examined. Microscopically, unattached hyphal segments are found. These are curved and short, each segment about 3 to 4 microns in diameter by 8 to 12 microns in length. Clusters of blastospores are also present. These are sufficiently larger, 4 to 6 microns in diameter, than *C. albicans* to permit easy differentiation. *M. furfur* cannot be cultured on artificial media.

6. The Cutaneous Monomorphic Molds

6-1. These fungi are the dermatophytes that generally infect only the hair, skin, and nails of man and animals. They have a particular affinity for keratinized tissue of the skin and its appendages but will also grow on media which is lacking in keratin. Present indications are that other body tissues may possess substances which are inhibitory to growth of these fungi.

6-2. All the dermatophytes were considered until recently to belong exclusively in the class *Deuteromycetes* or fungi imperfecti. Demonstration of ascospores in *Keratinomyces ajelloi* and *Microsporum gypseum* indicates that some of the dermatophytes are actually in the class *Ascomycetes*. Until further discoveries are made, we will consider that the majority of dermatophytes belong in the class *Deuteromycetes*.

6-3. Identification of the dermatophytes cannot be based solely on macroconidial morphology, since some strains produce these structures only rarely, while others never produce macroconidia. Also, mutant strains vary greatly in colony characteristics and pigmentation. For these reasons, physiological criteria may be more important than morphology in the identification of some members of this group. The more common physiological tests usually consist of nutritional tests to determine the need for special growth factors, optimal temperature requirements, and determination of the type of hair digestion "in vitro." The dermatophytes grow very well on a standard medium, such

TABLE 12
HUMAN CUTANEOUS MYCOSES AND ETIOLOGICAL AGENTS

Tinea Capitis (scalp)	*Tinea Barbae* (beard)	*Tinea Corporis* (body)	*Tinea Cruris* (groin)	*Tinea Pedis* (feet)	*Tinea Unguium* (nail)
Agents	Agents	Agents	Agents	Agents	Agents
M. audouinii [1] *M. canis* *M. distortum* *M. ferrugineum* *M. gypseum* *T. megninii* *T. mentagrophytes* *T. schoenleinii* [3] *T. tonsurans* [1, 4] *T. violaceum* [4]	*T. megninii* *T. mentagrophytes* *T. rubrum* *T. verrucosum*	*M. audouinii* *M. canis* *M. distortum* *M. ferrugineum* *M. gypseum* *T. concentricum* *T. mentagrophytes* *T. rubrum* *T. schoenleinii* *T. tonsurans* *T. verrucosum* *T. violaceum*	*C. albicans* *E. floccosum* *T. mentagrophytes* *T. rubrum*	*C. albicans* *E. floccosum* *T. mentagrophytes* [2] *T. rubrum* [2]	*C. albicans* *E. floccosum* *T. megninii* *T. mentagrophytes* *T. rubrum* *T. schoenleinii* *T. violaceum*

[1] Agent of epidemic ringworm.
[2] Most common cause of athlete's foot.
[3] Agent of favic ringworm (tinea favosa).
[4] Agent of black-dot ringworm.

TABLE 13
MAJOR CHARACTERISTICS OF THE DERMATOPHYTES

Genus	*Infected Areas*	*Host Preference*	*Hair Invasion*	*Wood's U-V Light Fluorescence*	*Usual Conidia Characteristics*
Microsporum	Skin, hair, and rarely the nails	*Primarily* children below age of puberty, also adults	Ectothrix—mosaic pattern of spores on hair shaft	Usually positive—exception: sometimes negative for *M. gypseum*	Many macroconidia (echinulated, thick-walls), few microconidia
Trichophyton	Skin, hair, and nails	Children and adults	Ectothrix, endothrix, and favic-endothrix	Usually negative—exception: sometimes positive for *T. tonsurans* or *T. schoenleinii*	Few macroconidia (smooth, thin walls), many microconidia
Epidermophyton (E. floccosum)	Skin and nails	Children and adults	None	Not applicable	Many macroconidia in clusters (smooth, thin walls), no microconidia

as Sabouraud's dextrose agar. The culture growth patterns on this medium for the common dermatophytes are shown in detail F of foldout 1. As we discuss each organism, refer to this foldout and study the applicable characteristics. The addition of chloramphenicol and cycloheximide have not demonstrated any inhibitory effect on their growth and so may be incorporated in the medium in the prescribed strength. With the addition of these antibiotics to retard growth of contaminants, the pH of Sabouraud's dextrose agar may be elevated to a range of 6.8 to 7.0 which is optimal for growth of the dermatophytes. The ideal temperature range for their multiplication is between 25° C. and 30° C.

6-4. While some dermatophytic species are found worldwide, others may be limited to a very small geographic area. Three broad categories, shown in table 11, are used to classify these fungi: *Anthropophilic* dermatophytes usually only infect man; *zoophilic* dermatophytes infect lower animals as well as man; and *geophilic* dermatophytes, which are saprophytes whose natural habitat is the soil. The last group does, however, infect man from time to time. Global distribution of these fungi is probably influenced a great deal by host preference and natural habitat.

6-5. The diseases caused by the dermatophytes are called tineas, or ringworm, because the lesion normally spreads out in a circular manner from the initial point of infection. The word "tinea" means "worm" in latin, and early workers felt these round lesions were caused by worms. A ringworm infection of the body is called tinea corporis; the feet, tinea pedis; the scalp, tinea capitis; the groin, tinea cruris; the nails, tinea unguium; as shown in table 12. Fungal infection resembling tinea unguium of the nails may also be caused by *C. albicans*. We will discuss members of the Genera *Microsporon, Trichophyton, Epidermophyton,* and *Keratinomyces* from the standpoint of clinical aspects and laboratory diagnosis. The major characteristics of the most common determatophytes are shown in table 13.

6-6. **Microsporum Audouinii.** *M. audouinii* is the most important causative agent of tinea capitis among grade school children in the United States. The disease affects boys more readily than girls in a ratio of about 3:1 or 4:1. It is an anthropophilic, or "man-loving" fungus, and in those rare cases involving animals the disease is mild and short lived. The infection is spread readily from child to child, either by direct contact or through the communal use of hats, combs, or brushes. One of the most important modes of transmission has been found in barber shops, where unsanitary equipment results in rapid dissemination of the disease.

6-7. The primary lesion occurs on the scalp, frequently at the hairline or in the area of the part. Usually, very little inflammation, erythema, or kerion formation accompanies the disease; the usual symptoms are hair loss and scaling. A kerion is a raised mass of tissue, suppurating at numerous points. Generally, the disease demonstrates well-defined patches of scaliness and hair loss, but it is not uncommon for scattered single hairs to be infected. *M. audouinii* invades the hair in a characteristic manner causing what is referred to as small-spored ectothrix invasion. The spores are formed from mycelia which have developed within the hair, broken through to the outer surface, and then fragmented into a sheath of tightly packed arthrospores. When the symptoms include much tissue inflammation and kerion formation, the disease course is generally short, or about 2 to 4 weeks in duration. More commonly, when the infection causes only hair loss and scaling it may heal spontaneously in 3 or 4 months. Some cases linger on for 2 to 3 years, however.

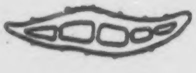
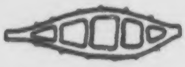
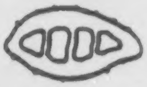
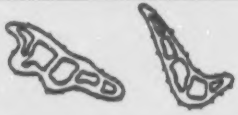
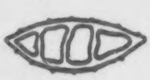
6-8. In laboratory identification we examine the scalp of the patient for hair loss, scaling, erythema, and/or kerionic lesions. The scalp should be scanned under the ultraviolet light of a Wood's lamp in a dark room, paying particular attention to any suspicious areas previously observed in daylight. If *M. audouinii* is present, even only on a few hairs, it will fluoresce a bright yellowish-green. But it is well to note that some strains of *M. audouinii* will not fluoresce when exposed to ultraviolet light. In early infections, where only the base of the hair is involved, it may be necessary to pluck the hair and examine it under the Wood's lamp to detect fluorescence.

6-9. Skin scrapings or hair should be collected from the margin of the lesion. When ultraviolet light is used, hair which fluoresces should be plucked and mounted in 10-percent potassium hydroxide. Microscopically, a sheath of small spores in mosaic completely surrounds the hair at its base; mycelium is found running lengthwise within the hair. Mycelium and chains of arthrospores are found in skin scrapings. On Sabouraud's dextrose agar containing chloramphenicol and cycloheximide the following colonial characteristics will be observed. The colony is slow-growing, flat, and velvety with whitish-tan to brownish surface. The reverse pigment if present, may be light salmon, or orange-tan. The growth of *M. audouinii* on rice grain medium is very poor, as compared to the other species of *Microsporum*. This relationship is shown in table 14. Microscopically, in wet mounts from culture, the mycelium is usually sterile (Pectinate) with many chlamydospores present. The rare microconidia are clavate in shape and borne sessile on the hyphae. Some strains of *M. audouinii* may form small numbers of macroconidia but these are bizarre-shaped and of little diagnostic value.

6-10. **Microsporum Canis.** *M. canis* is primarily an animal parasite. Because it is the most common cause of ringworm in cats and dogs throughout the world, it is classified as a zoophilic fungus. Human infections, especially in children, are not unusual. They generally result from contact with infected puppies or kittens. It is commonplace for several or all the members of a family to become infected; however, it is difficult to determine whether the organism was contracted from a common source or resulted from human to human transfer. About 10 percent of tinea capitis infections in the U.S. are due to *M. canis*. This percentage may run as high as 65 percent in specific areas, probably reflecting the disease incidence in the local dog and cat population. The

TABLE 14

THE GROWTH OF MICROSPORUM AUDOUINII AS COMPARED TO OTHER SPECIES OF MICROSPORUM

Organism	Growth Pattern on Rice Grains	Characteristic Macroconidium, Sketch
M. audouinii	negligible growth, producing brown coffee-stain discoloration on rice grains.	
M. canis	good growth, white cottony mycelium producing many characteristic macroconidia.	
M. cookei (saprophytic)	good growth, white cottony mycelium, usually not necessary for identification.	
M. distortum	good growth, white cottony mycelium producing many characteristic macroconidia.	
M. gypseum	good growth, white granular appearance resembling finely ground gypseum.	

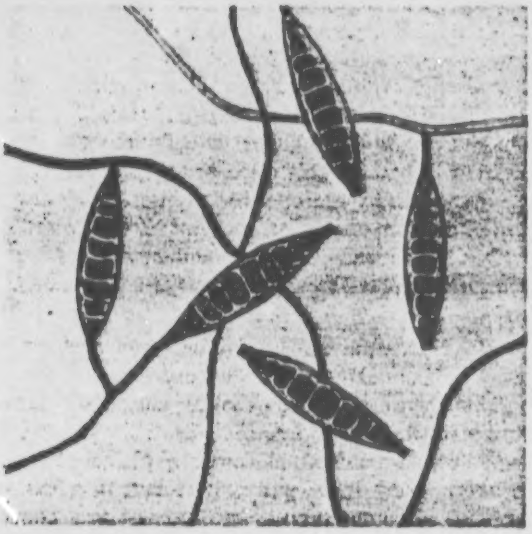


Figure 15. Microsporum canis (macroconidia).

infection may appear on any skin surface of the body; and it is usually inflammatory, with development of kerions and much suppuration. When this is the case spontaneous healing usually takes place after 3 to 4 weeks. In the chronic form with little or no inflammation healing may require many months.

6-11. The patient is examined first for hair loss and skin lesions, which may be dry and scaly or suppurative and kerionic in nature. *M. canis* causes a small-spored ectothrix invasion of hair; and as in the case of *M. audouinii*, it fluoresces a bright yellow-green under UV light. *M. audouinii* and *M. canis* cannot be differentiated on the basis of clinical symptoms or hair examination. Cultural studies are required. A potassium hydroxide mount of an infected hair or skin scrapings will result in findings identical to those elaborated for *M. audouinii*.

6-12. Growth on Sabouraud's dextrose agar with antibiotics is rapid; and the colony will at first be white and silky with a bright yellow marginal pigment. As the colony ages its surface becomes dense, tan and cottony, sometimes in irregular tufts or concentric rings. On rare occasion there will be no reverse (underside) pigmentation; but usually a bright yellow, pigment is present, and it gradually turns dull orange-brown. *M. canis* grows well on rice grains. Microscopically, macroconidia are numerous in wet mounts, 8- to 15-celled, and spindle-shaped. They often terminate in a distinct knob and have thick echinulated walls, as shown in figure 15. The microconidia are few in number, clavate, and usually borne sessile on the hyphae.

6-13. **Microsporum Gypseum.** *M. gypseum* is a geophilic fungus, and therefore the majority of infections of both man and animals may be traced to the soil. Human infections are rare in spite of the fact that recovery of this fungus from soil samples is very common. Generally, people with ringworm infections develop a single lesion on an exposed area of skin or the scalp. Considerable tissue reaction is present, and the development of kerions is not uncommon. When these areas heal, scarring and permanent hair loss are a frequent result.

6-14. A preliminary examination of the patient for skin lesions and hair loss is made. Lusterless, brittle hairs are plucked for examination, and skin scrapings are taken from the margin of the infected area. Examination of the hairs in direct potassium hydroxide mount reveals a large spored (5 to 8 microns) ectothrix type invasion, with arthrospores in chains or irregular masses on the air surface. Mycelium runs lengthwise within the hair. Similarly mounted skin scrapings show masses of arthrospores, occasionally in chains, and mycelium. Hairs infected with *M. gypseum* either fluoresce very poorly or not at all, so a Wood's lamp is of little value. This organism grows well on rice grain medium.

6-15. Cultural studies are essential for identification. Growth on Sabouraud's dextrose agar with antibiotics is rapid. The colony is flat, with irregular fringed borders and a coarsely powdery surface ranging from light ochre to deep cinnamon brown. Tufts of white, fluffy, sterile growth develop rapidly on the colony surface, and the reverse pigment is usually a dull yellow to tan, or rarely pinkish to red. Microscopic examination of a wet

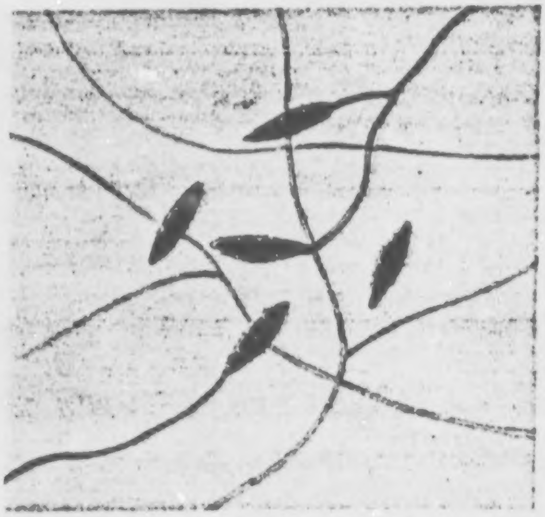


Figure 16. Microsporum gypseum (macroconidia).

mount illustrated in figure 16 shows numerous macroconidia, 3- to 9-celled, ellipsoid, shorter and broader than those of *M. canis*, with thinner, echinulated walls. The microconidia are rare, clavate, and usually borne sessile on the hyphae.

6-16. **Genus Trichophyton.** The members of the Genus *Trichophyton* are the most important causative agents of ringworm of the feet and nails throughout the world. They sometimes cause tinea infections on other body areas, such as the trunk,

TABLE 15A
CULTURAL CHARACTERISTICS OF MORPHOLOGY OF DERMALOPHYTES

Organism	*Key to Plate II*	*Growth Rate*	*Texture*	*Topography*	*Surface Pigment*	*Back (reverse) Pigment*
E. floccosum	A	Slow	Powdery	Flat or heaped, folded in center	Olive green to tan	Yellow to tan
K. ajelloi	B	Rapid	Powdery	Flat	White, cream to pink	Purple, black or absent.
M. audouinii	C	Slow	Velvety	Flat	Cream to tan	Pink to brown
M. canis	D	Rapid	Cottony	Flat	White to yellow	Translucent yellow or absent.
M. cookei	E	Rapid	Flat, central growth	Powdery to granular	White to pink	Red to deep purple
M. distortum	F	Rapid	Velvety	Flat	Cream to tan	Tan
M. gypseum	G	Rapid	Powdery to granular	Flat	Tan to cinnamon brown	Yellow to tan
T. mentagrophytes	H	Rapid	Velvety, powdery, or granular	Flat	Usually white, cream, or tan; rarely pink, red, yellow, or orange	Usually rose to brown or colorless, rarely yellow to orange
T. rubrum	I	Slow	Usually cottony, rarely powdery	Flat, rarely folded	Usually white; rarely rose	Usually deep red; rarely colorless
T. schoenleinii	J	Slow	Moist and glabrous, or powdery to velvety	Usually heaped and folded; rarely submerged	Usually white, cream, or tan; rarely brown	Tan or absent
T. tonsurans	K	Rapid	Usually velvety to powdery, rarely granular	Usually heavily folded with depressed centers; rarely flat	Tan, white, yellow, rose, lavender, or brown	Yellow, red, brown, purple, or absent
T. verrucosum (*T. faviforme*)	L	Very slow, stimulated by thiamine 37° C.	Moist and glabrous, powdery or velvety	Usually heaped and folded, rarely flat	White, tan or yellow	Yellow or absent
T violaceum	M	Slow	Moist, glabrous to leathery; or velvety; rarely cotton	Finely wrinkled	Cream, then violet to purple, or colorless	Purple or absent
T. concentricum [1]		Slow	Glabrous, downy areas	Heaped and folded, often cracked	White, tan or brown	Yellow
M. ferrugineum (*T. ferrugineum*)		Slow	Moist and glabrous, or velvety	Finely wrinkled, or flat with radial grooves	Rust or white	Rust or colorless
T. megninii [1]		Rapid	Velvety	Flat; may have radial grooves	White to rose	Deep rose

---

[1] Rare etiological agents in North America.

NOTE: Rare Trichophyton spp. not included in this manual include *T. equinum*, *T. gallinae*, *T. terrestre*, and *T. yaoundei*.

TABLE 15B
MICROSCOPIC MORPHOLOGY OF DERMALOPHYTES

Organism	*Key Figure*	*Production of Macroconidia*	*Shape of Macroconidia*	*Thickness of Macroconidial Wall*	*Surface of Macroconidial Wall*	*Microconidia*
E. floccosum		Abundant in clusters	Clavate	Thin	Smooth	Unknown
K. ajelloi		Abundant	Elliptical	Thick	Smooth	Pyriform, numerous or rare
M. audouinii		Rare or absent	Irregular to spindle-shaped	Thick	Echinulated	Usually few; rarely numerous
M. canis		Abundant	Spindle-shaped	Thick	Echinulated	Present
M. cookei		Abundant	Elliptical	Thickest of *Microsporum*	Echinulated	Present
M. distortum		Abundant	Bizarre, almost any shape	Thick	Echinulated	Present
M. gypseum		Abundant	Elliptical	Thin	Echinulated	Present
T. mentagrophytes		Few to many	Clavate or variable	Thin	Smooth	Few to many, spherical
T. rubrum		Usually, few; rarely numerous	Pencil-shaped long	Thin	Smooth	Few to many, oval to elongate
T. schoenleinii		Unknown	----------	----------	----------	Rare, induced on enriched medium
T. tonsurans		Rare	Clavate to irregular	Thin	Smooth	Many; pyriform to elongate; large and irregular with age
T. verrucosum (*T. faviforme*)		Rare; induced on thiamine medium	Irregular	Thin	Smooth	Rare; induced on thiamine medium
T. violaceum		Rare, induced on thiamine medium	Clavate to irregular	Thin	Smooth	Rare; induced on thiaminemmedium
T. concentricum[1]		Unknown	----------	----------	----------	Rare, pyriform
M. ferrugineum[1] (*T. ferrugineum*)		Unknown	----------	----------	----------	Rare; induced on enriched medium
T. megninii[1]		Rare	Clavate to Narrow	Thin	Smooth	Pyriform, numerous or rare

---

[1] Rare etiological agents in North America.

NOTE: Rare *Trichophyton* spp. not included in this manual include *T. equinum, T. gallinea, T. soudanense, T. terestre,* and *T. yaoundei.*

scalp, or beard area of males. Spontaneous healing at puberty (as with microsporum infections) does not occur and as a result, adult infections are not uncommon. Infections with members of the Genus *Trichophyton* result in a great variation in tissue response, ranging from superficial scaling and hair loss to highly inflammatory lesions. For this reason, diagnosis on the basis of the clinical symptoms is highly unreliable.

6-17. Various members of the Genus *Trichophyton* attack the hair in differing manner, thereby aiding somewhat in their differentiation. Diagnosis is none the less dependent on isolation and identification. Many species of *Trichophyton* have been described, but the likelyhood is great that a substantial number of these were variants of a single species. On the basis of current studies twelve distinct species have been described; the methods used for identifying the more common trichophyton disease agents from the standpoint of preliminary diagnosis and cultural and microscopic characteristics are elaborated in tables 15A and 15B, respectively.

6-18. As stated above, the manner of "in vivo" hair invasion can be helpful in differentiating the *Trichophyton spp. T. mentagrophytes, T. equinum,* *T. verrucosum* and *T. rubrum* all cause an ectothrix type invasion with arthrospores grouped on the outside of the hair shaft. *T. tonsurans* and *T. violaceum* cause endothrix type hair infections in which the arthrospores are found only within the hair shaft. The hair in this type invasion becomes very fragile due to swelling. It breaks off close to or below the scalp surface. The resulting black, speckled appearance has given rise to the term "black-dot" ringworm. Light-haired individuals fail to demonstrate this characteristic. One final type of hair invasion called "favic" is characteristic of *T. schoenleinii*. In contrast to the previous types, the hair is filled with mycelium, and only rarely is an arthrospore seen. The hair does not become fragile and often does not break off. It is not unusual to see empty spaces where mycelium has degenerated. The spaces may become filled with air bubbles and fluid when wet mounts are prepared. Mutant strains of dermatophytes other than *T. schoenleinii* may also produce a favic type of hair invasion.

6-19. Some species of *Trichophyton* have demonstrated an ability to grow in two distinct forms: "downy" variety and "granular variety." The downy variety of *T. mentagrophytes*, or *T. mentagrophytes* var. *interdigitale*, has a relatively low virulence in man and is normally associated with chronic tinea pedis. The "granular variety" of animal origin is highly virulent, causing inflammatory suppurative ringworm. *T. rubrum* as well as other *Trichophyton* species also have this ability to increase their virulence by animal transfer. Nutritional tests using growth requirements for vitamins and amino acids are helpful in differentiating *Tri-*

TABLE 16

GROWTH PATTERNS FOR TRICHOPHYTON

Dermatophyte		*Physiological Test Media*			
		Casein		Casein + nicotinic acid	
T. equinum		0		4+	
T. mentagrophytes		4+		4+	
		Casein		Casein + thiamine	
M. ferrugineum		4+		4+	
T. mentagrophytes		4+		4+	
T. rubrum		4+		4+	
T. tonsurans		± to 1+		4+	
T. violaceum		±		4+	
		NH_4NO_3		NH_4NO_3 + histidine	
T. gallinae		4+		4+	
T. megninii		0		4+	
		Casein	Casein + inositol	Casein + thiamine	Casein + thiamine and inositol
T. concentricum	50%	4+	4+	4+	4+
	50%	2+	2+	4+	4+
T. schoenleinii		4+	4+	4+	4+
T. verrucosum	85%	0	±	0	4+
	15%	0	0	4+	4+

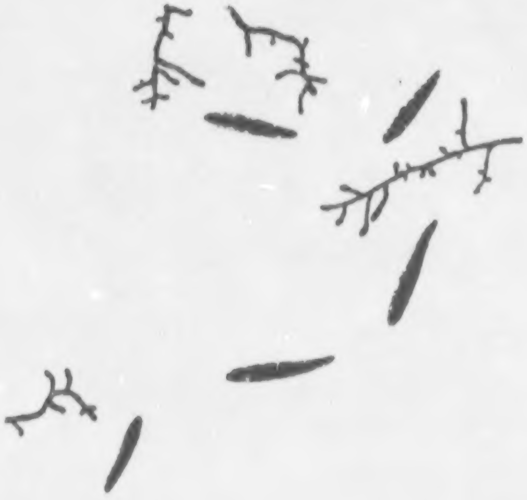


Figure 17A. Trichophyton mentagrophytes (Clavate Macronidia).

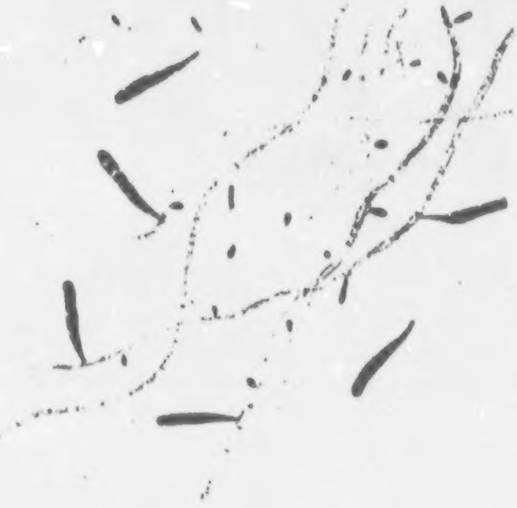


Figure 17C. Trichophyton rubrum.

chophyton species. Special media are available through commercial sources in dehydrated form, and the techniques used may be found in most mycology textbooks. The growth patterns on these media are described in table 16. If atypical isolates of *T. rubrum* or *T. mentagrophytes* are encountered, an "in vitro" hair culture may be used for separation of the two species. The test is based on ability to invade the hair. Varieties of *T. mentagrophytes* are capable of causing wedge-shaped perforations of the hair, while varieties of *T. rubrum* will not perforate the hair. The hair will usually not show perforations until the 10th to 14th day, and the test should not be considered negative until 28 days have elapsed. The steps in this technique may also be found in most up-to-date clinical mycology textbooks. Characteristic microscopic morphology of the more common genera and species of *Trichophyton* are illustrated in figures 17A thru F.

6-20. **Epidermophyton Floccosum.** *E. floccosum* is one of the more common etiological agents of tinea cruris (ringworm of the groin), and tinea pedis (ringworm of the feet). It is, however, well below *T. rubrum* and *T. mentagrophytes* in incidence as the agent of these dermatophytic diseases. It has also been isolated on occasion as the

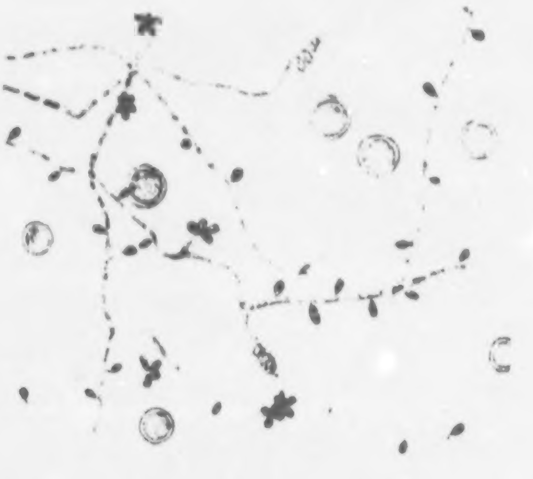


Figure 17B. Trichophyton (En thyrse and En grappe formation).

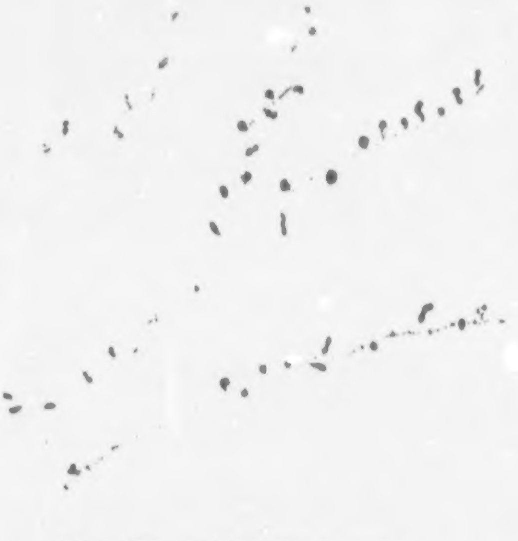


Figure 17D. Trichophyton tonsurans.

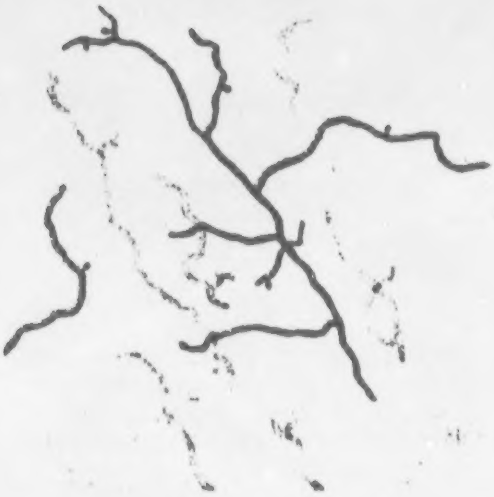


Figure 17E. Trichophyton violaceum.

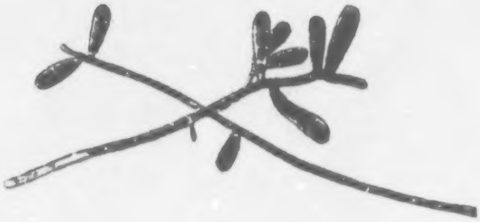


Figure 18. Epidermophyton floccosum (macroconidia).

etiological agent of tinea unguium. *E. floccosum* does not invade the hair and will not fluoresce under UV light.

6-21. Laboratory identification of *E. floccosum* is based on colonial and microscopic characteristics. Scrapings are collected from the periphery of skin lesions and examined in 10-percent potassium hydroxide for the presence of mycelium and chains of arthrospores. Regardless of the findings, the scrapings should be cultured on Sabouraud's dextrose agar with or without antibiotics. Growth of *E. floccosum* is slow. At first it is fluffy white, gradually becoming velvety or powdery. The surface is flat or radially folded, and a tan to olive-green color develops. Reverse pigment is a yellowish tan. When growth is mature, teased mounts of *E. floccosum* will reveal numerous macroconidia: 2- to 4-celled, large, broad, blunt to clavate in shape, with smooth, thin walls. They may be borne singly or in groups of two or three. These groupings are shown in figure 18. Many chlamydo spores may be present and microconidia are absent.

6-22. **Keratinomyces Ajelloi.** This, dermatophyte and *M. gypseum* are the only geophilic fungi with global distribution. *K. ajelloi* has been isolated only on rare occasions from animals, and it is generally felt to be of little clinical importance. Since data regarding the prevalence of der-

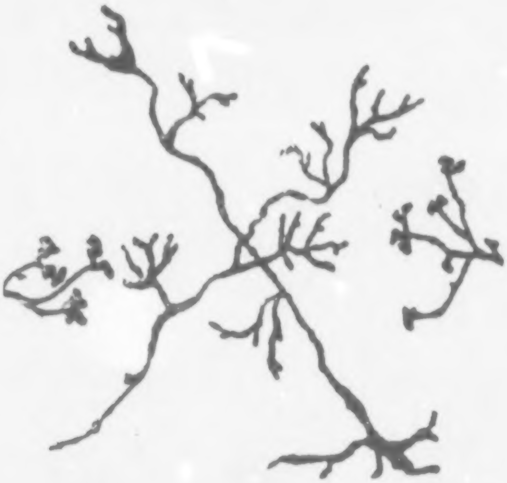


Figure 17F. Trichophyton schoenleinii.

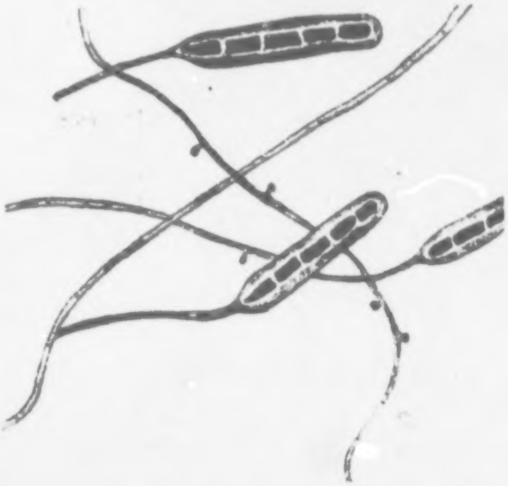


Figure 19. Keratinomycetes ajelloi (macroconidia).

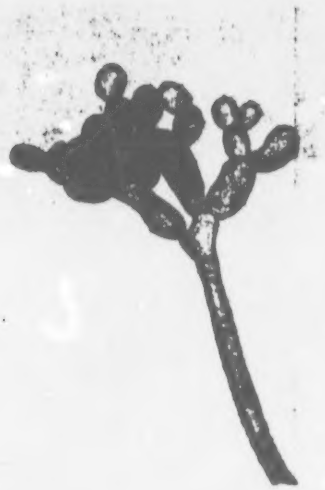


Figure 20A. Chromoblastomycotic—Cladosporum type.

matophytes in many regions of the world is scanty, and the etiological agents of many ringworm infections go unreported and possibly undiagnosed, it is important that the technician be able to isolate and identify this potential agent of cutaneous mycotic disease.

6-23. As with the previously discussed dermatophytes, suspect hair and skin scrapings are collected for KOH wet mount examination and for culturing. *K. ajelloi* does not fluoresce under ultraviolet light. The wet mount of the hair may demonstrate mycelium within the shaft without a distinct sheath of spores around the hair. Skin scrapings in wet mount may reveal mycelium and chains of arthrospores. When cultured on Sabouraud's dextrose agar with or without antibiotics colony growth is rapid and either flat or somewhat heaped or folded. The surface is finely powdery, or downy, and cream to tan or orange-tan in color. Characteristically, areas of white fluffy growth develop rapidly, and the reverse pigmentation may be either a deep bluish-black or absent.

6-24. Microscopic examination of a teased mount from culture will demonstrate many macroconidia. They are long and slender with parallel walls tapering at each end (cylindro-fusiform), 8 to 12 cells are present. The macroconidial walls are wide (thicker than those of *M. canis)* and have a smooth surface, as shown in figure 19. Microconidia are abundant in some strains, while rare in others. When present, they are ovate to pyriform in shape and borne sessile on the hyphae.

7. The Subcutaneous Monomorphic Molds

7-1. This group of fungi, as the heading implies, produces disease in both the cutaneous and subcutaneous tissues. Members have on rare occasion penetrated to the deep organs of the body.

7-2. **Chromoblastomycotic Fungi.** These are the etiological agents of chromoblastomycosis. Like the other agents of subcutaneous mycotic diseases, in all probability they lead a saprophytic existence in nature. Most human or animal infections are traceable to wounds contaminated with soil or vegetative matter. Embedding of the chromoblastomycotic fungal agent in the cutaneous or subcutaneous tissues results. The disease seems to be more common in tropical and subtropical areas of the world, but at least 24 cases have been re-

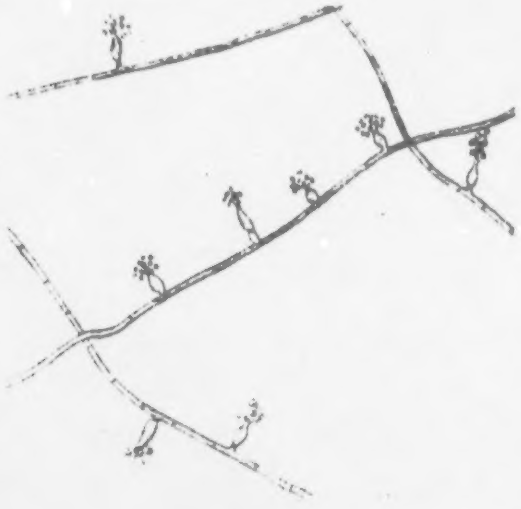


Figure 20B. Chromoblastomycotic—Phealophora type.

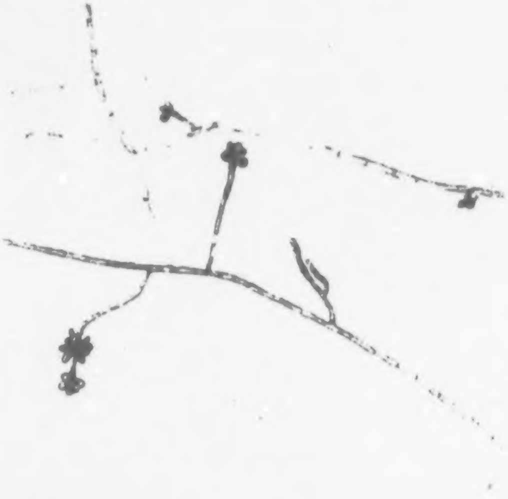


Figure 20C. Chromoblastomycotic—Acrotheca type.

ported in the United States. The disease itself produces no specific pigment, and the organisms do not multiply in the tissues by a true budding process. Hence, the name chromoblastomycosis is a misnomer. At present, however, this term continues to be used for this particular type of mycosis.

7-3. There are three types of conidiophore formation demonstrated by the various species causing chromoblastomycosis. The ability to differentiate them will aid appreciably in definitive identification. In the cladosporium type, formally called hormodendrum type, the conidiophore is a simple stalk slightly enlarged at its distal tip. Conidia are produced at this distal tip, forming chains which branch if a single conidium forms two or more buds. This type of sporulation is shown in figure 20A. These chains are often broken up in teased-mount preparations, and under high magnification scars may be seen at their former points of attachment. These darkened scars are referred to as disjunctors. A second type of sporulation, shown in figure 20B and referred to as the phialophora type is characterized by the development of highly distinctive conidiophores called phialides which may develop terminally or along the mycelium. These phialides are flasklike structures having a large round or oval base, a constricted neck and a cup-shaped outlet which may flare out to form a distinct lip. Conidia are developed endogenously within the base and extruded through the outlet. They accumulate in a spherical mass of loosely adherent spores. A final type of sporulation (see fig. 20C) called acrotheca type is characterized by the formation of conidia along the sides of irregularly club-shaped conidiophores. The conidia are usually single, but on occassion may show a tendency to form chains. When the conidia become detached, as occurs in the preparation of a teased mount, the conidiophores present a roughened, irregular surface due to scarring. These scars represent the former points of attachment of the conidia on the conidiophore.

7-4. The fungi responsible for chromoblastomycosis are *Phialophora verrucosa*, *Cladosporium carrionii*, *Fonsecaea pedrosoi* and *Fonsecaea compacta*. The characteristics of the disease are the same regardless of which of these fungi is the etiological agent, and therefore they will be discussed collectively. The infection usually begins as a papule or pustule at the site of initial penetration. This persists for a long time, and gradually enlarges and becomes ulcerated. Other lesions may develop by direct extension of the primary lesion, additional implantations or via the lymphatic system. For some little understood reason, the disease tends to remain localized in the limb or area initially infected. Eventually, large masses protruding several centimeters above the skin level will develop. It is not uncommon for these masses to be attached by a narrow pedicle. Bacterial invasion is quite common, causing purulent, foul smelling crusting of the lesions. In advanced cases lymphatic stasis and elephantiasis are quite common.

TABLE 17

LABORATORY IDENTIFICATION OF CHROMOBLASTOMYCOTIS SPECIES AND RELATED SAPROPHYTIC SPECIES

Organism		*Macroscopic Morphology*	*Microscopic Morphology*	*Types of Sporulation*	*Liquifaction of Gelatin*	*Hydrolysis of Loeffler's Serum Slant*
Cladosporium carrionii		Slow growing; dark, greenish-black.	Chains of spores are long and branching; appear similar to those of the saprophytic Cladosporium sp.	Cladosporium	0	0
Fonsecaea compacta		Slow growing; dark, greenish-black.	Spores are not elongate as in other species but are nearly round and occur in closely packed short chains; cladosporium type sporulation predominates.	Acrotheca, cladosporium, and phialophora.	0	0
Fonsecaea pedrosoi		Slow growing; dark, greenish-black.	Chains of spores are usually short. Cladosporium type sproulation usually predominates. Acrotheca and phialophora types of sporulation are rare in most strains. Occasional strains show largely acrotheca type sporulation.	Acrotheca, cladosporium, and phialophora.	0	0
Phialophora verrucosa		Slow growing; dark, greenish-black; occasional strain produces a soluble brown pigment.	Sporulation may occur on aerial mycelium or in the depths of the medium.	Phialophora	0	0
Cladosporium species (saprophyte)		Rapid growing; dark, greenish-black.	Chains of spores are long and branching.	Cladosporium	+	+

These masses, especially on the feet, may become so enlarged they resemble small cauliflowers.

7-5. A few cases of chromoblastomycosis with central nervous system involvement have typical skin lesions. These presumably are the result of blood stream dissemination. Other cases, however, show no skin lesions, indicating that some internal organ, possibly the lung, was the initial, unrecognized focus of infection.

7-6. In the laboratory diagnosis of chromoblastomycosis scrapings, scales, or exudate are examined in potassium hydroxide mount for the presence of small clusters of spherical dark brown, thick-walled cells that reproduce by equatorial splitting (not budding). The abundance of organisms in the clinical material makes direct examination a rapid means of diagnosis. The material should be cultured on Sabouraud's dextrose agar with antibiotics and incubated at room temperature. The fungi causing chromoblastomycosis all produce dark colonies which are heaped or sometimes slightly folded, and typically slow growing. The colonies are covered with a short aerial mycelium which forms a greyish velvet nap, while the reverse pigmentation is a jet black.

7-7. A teased mount should be examined microscopically for the type, or types, of sporulation. In species demonstrating multiple types of sporulation, determination of the predominating form can be useful in accurate identification. Table 17 contains data regarding the colonial morphology, microscopic morphology, and physiological characteristics of the agents of chromoblastomycosis and the closely related saprophytic *Cladosporium* species. Histopathologically, chromoblastomycosis may be readily diagnosed in tissue sections stained with hematoxalin and eosin, or by use of the periodic-acid-Schiff (PAS) staining procedure. The fungi causing the disease all appear identical in tissue, and in potassium hydroxide wet mounts of scrapings or exudate. The sclerotic bodies stain dark red in a PAS preparation. Animal inoculation and serological tests are not used in diagnosing this disease.

7-8. **Maduramycotic Fungi.** In 1842, McGill described the disease from an area around Madura, India, although the condition had been found in this geographic location for many years prior to that date.[1] Eventually it was labeled "Madura foot." Vandyke Carter in 1874 was the first to describe changes in the subcutaneous tissue and bones, and to identify fungal elements in the grains obtained from within the sinus tracts of affected areas. Since parts of the body other than the foot may be infected, Carter introduced the term "mycetoma," meaning fungus tumor.

7-9. The disease has global distribution, but it is considerably more prevalent in tropical and subtropical areas. The fungal agents responsible for this disease are saprophytic in soil and vegetation and gain entry either by a scratch or a penetrating wound. The greater incidence in hot, humid climates can be related to the fact that the causative fungi flourish under these environmental conditions and the inhabitants are more likely to go barefoot.

7-10. The disease (maduromycosis) is chronic, sometimes lasting a lifetime. It may affect any part of the body although foot infections are the most common. After gaining entrance, the responsible mycotic agent invades the subcutaneous tissue and eventually muscle, bone, and other surrounding tissue become involved. The region affected becomes one in which acute purulent abscesses are continuously developing, burrowing in several directions, and eventually discharging to the surface. Localized areas of inflammation and swelling appear as new abscesses form, while older areas demonstrate healing by dense fibrosis. When the foot is involved, it becomes tender and painful and loses most of its original contour because of swelling. Draining sinuses cover the surface, exuding pus-containing granules, which are readily seen. These granules may be white, yellow, red, or black in color, depending on the fungal agent. They may attain a size of 2 to 3 mm. An experienced mycologist can often identify a specific maduromycotic fungal agent on the basis of the structural elements of the granules produced.

7-11. Other fungi, such as *Actinomyces spp.* and *Nocardia spp.* cause mycetomas; however, the mycelia are considerably smaller in diameter (less than 1 micron), and they do not produce the large numbers of chlamydospores normally found in maduromycotic infections.

7-12. The fungi responsible for this disease are *Allescheria boydii, Cephalosporium falciforme, Madurella grisea, Madurella mycetomii* and *Phialophora jeanselmei*. With the exception of *Cephalosporium falciforme*, cultures of these fungi are shown in detail A of foldout 2 (separate inclosure). Diagnosis of one of these agents of maduromycosis normally begins with examination of pus, currettings, or tissue biopsy material for the presence of "granules." These granules, which may vary in size between 0.5 and 2 mm., are first examined grossly in potassium hydroxide or water, and such characteristics as color, texture, shape, and size carefully noted. A granule should be crushed between two slides and studied microscopically. It is essential at this point to distinguish maduromycotic granules, which contain a mycelium between 2 to 4 microns in diameter and numerous chlamydospores, from actinomycotic granules which contain a mycelium of less than 1 micron in diameter and no chlamydospores. Refer to table 18 for a comprehensive description of the granules produced by the agents of maduromycosis.

---

[1] Wilson and Plunkett, *The Fungous Diseases of Man, Second* Printing, 1967, page 150.

7-13. The contents of the granules or material aspirated from unopened lesions, should be cultured on Sabouraud's dextrose agar containing antibacterial agents. Cycloheximide should not be used as some of the fungi which cause maduromycosis are sensitive to this antibiotic. Prior to culturing, wash the granules in physiological saline containing a combination of either penicillin and streptomycin (5,000 units/ml.) or chloramphenicol (0.05 mg./ml.). The granules may be left overnight in this solution prior to culturing. Several tubes of media should be inoculated in order to permit incubation at both 37° C. and room temperature. Identification of these fungi is based on their morphological and physiological characteristics which are available in table 19.

8. The Systemic Monomorphic Molds

8-1. These organisms which produce deep-seated human infections have a taxonomic position which differs appreciably from that of fungi previously considered. In order to clarify these differences, reference should be made to figure 21 and comparison made to figure 2, Chapter 1.

8-2. The fungi which will be discussed in this section include the genera *Actinomyces*, *Nocardia* and *Streptomyces*. They are grouped according to Bergey's manual with the bacteria in the class, *Schizomycetes*. These organisms have an irregular morphology, and they occasionally produce branching forms which are generally considered as rudimentary mycelium—and in some cases true mycelium. This mycelium is quite thin, 1 micron or less in diameter, and in one family, the *Actinomycetaceae*. There is a tendency to fragment quite readily into bacillary and coccoid forms which are extremely difficult to distinguish from bacterial cells. Their close relationship to the bacteria is pointed up by the fact that, like the bac-

TABLE 18

AGENTS OF MADUROMYCOSIS

Species	Appearance in Tissues						
	Morphology of Granules						
	Color	Size	Shape	Texture	Contents	"Cement	Clubs
Allescheria boydii	White or yellowish	500μ	Round or lobulated	Soft	Hyaline hyphae up to 5μ D. Chlamydosopores 3–20μ D., especially at the periphery.	0	0
Cephalosporium falciforme	white or yellowish	1–1.5 mm	Irregular	Soft	Hyaline hyphae up to 3–4μ D. Chlamydospores up to 12μD.	0	0
Madurella grisea	black	1mm or larger	Round or lobed	Soft at first, become hard & brittle	Unpigmented central zone contains hyaline hyphae. Periphery of large brown cells in band of dark brown "cement"	Dark brown "cement" in periphery only	0
Madurella mycetomii	black	1 mm	Round or lobed	Firm at first, Later hard and brittle	Light brown or hyaline hyphae 1–5μ D. Chlamydospores up to 25μ. Abundant at periphery. Brown particles in hyphae and chlamydospores.	Hyphae embedded in brown granular "cement"	0
Phialophora jeanselmei	black	Variable	Irregularly round to vermicular with hollow center or crescent to helicoid	Soft	Largely dark brown chlamydospores 5–10μ. Occasionally brown hyphae.	0	0

TABLE 19

AGENTS OF MADUROMYCOSIS: MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS

Organism	*Macroscopic Morphology*	*Microscopic Morphology*	*Physiological Characteristics*			
			Optimum Temperature	*Proteolytic Activity*	*Amylolytic Activity*	*Carbon Assimilation*
Allescheria boydii	Colony rapid-growing. Fluffy aerial mycelium, at first white, later dark brownish-gray. Reverse of colony gray to black. Rarely membranous ivory-colored colonies.	Hyphae hyaline 1-3μ D. Conidia unicellular grayish-brown, borne singly, pyriform 4-9/6-10μ in clusters or coremia. Cleistothecia with 8 spored asci may be present.	30°-37° C...	+	+	Maltose 0
Cephalosporium falciforme	Colony slow-growing, fluffy or tufted, lavender, buff, or pinkish. Reverse currant red. May produce a soluble pigment.	Hyphae hyaline 2-4μ D. Conidia sickle-shaped borne successively in terminal clusters from simple conidiophores.	30° C......	Weak	0	Maltose +
Madurella grisea	Colony slow-growing with tan or gray velvety aerial mycelium over a brown mat.	Hyphae of two types: pale thin cylindrical 1-3μ or dark thick "toruloid" made up of chains of budding cells 3-5μ. Chlamydospores rare. Conidia, sclerotia, or hyphal inclusions absent.	30° C......	Weak	+	Sucrose +
Madurella mycetomii	Colony slow-growing flat or folded membranous or fluffy white, yellow, or yellow-brown.	Hyphae 1-4μ with numerous chlamydospores up to 25μ D. Some strains produce conidia from tiny flask-shaped phialides. Black sclerotia, up to 750μ sometimes produced.	37° C......	Weak	+	Sucrose 0
Phialophora jeanselmei	Colonies slow-growing, black, at first moist with skin-like surface, becomes covered with grayish velvety aerial mycelium. Black on reverse.	Hyphae at first "toruloid" made up of chains of budding cells, later becomes straight and regular. Conidia produced in tube-like phialides. Pullularia-like sporulation also described.	30° C......	0	0	Sucrose +

NOTE: *A. boydii*, *C. falciforme*, and *P. jeanselmei* can usually be identified on the basis of their gross and microscopic morphology. The lack of proteolytic activity of *P. jeanselmei* serves to separate this fungus from the saprophytic *Cladosporium* species which have similar colonies.
M. mycetomii and *M. grisea* are identified on the basis of both their morphological and physiological characteristics.

teria, they cause suppurative tissue reactions and are sensitive to many of the same antibiotics. Another feature of the family *Actinomycetaceae* is a lack of chitin and cellulose in the cell walls. Bacteria lack these substances, while they are normal constituents of the walls of the *Eumycetes*, the true fungi. It has not been conclusively established whether *Actinomyces spp.* and *Nocardia spp.* are bacterial or fungal in nature. Although they were discussed briefly in Volume 2, Chapter 5, The Acid-Fast Bacilli, we will consider them as fungi for purposes of this work, since the diagnostic techniques used closely resemble those used for other mycotic agents.

8-3. **Clinical Aspects of Actinomycosis.** Actinomycosis is a worldwide chronic, suppurative, granulomatous infection which may exist in several well-defined clinical forms. The causative agents of the disease have never been isolated from any natural habitat outside the human body; however, their existence in saprophytic and parasitic form in man and animals is common. In tonsils removed by tonsillectomy, a significant number contain an anaerobic actinomycete. Anaerobic actinomycetes are common inhabitants of the tonsillar crypts, gingiva and teeth of apparently normal individuals. Therefore, the disease must be considered endogenous in origin.

8-4. Cervico-facial actinomycosis due to *Actinomyces israelii* frequently follows tooth extraction or fractures of the teeth-bearing areas. Usually, the primary site of infection is at the angle of the jaw, although the maxillary sinus is also particularly susceptible. After gaining entry the organisms spread to soft tissue and bone of the face and neck, causing much swelling and the development of abscesses which drain a purulent fluid. The fluid contains tiny yellowish-white friable masses of the causitive fungi, commonly referred to as "sulfur granules."

8-5. *Actinomyces bovis* causes a similar disease

KINGDOM—Plant
  SUBKINGDOM—Thallophyta
    PHYLUM—Schizomycophyta
      CLASS—Schizomycetes
        ORDER—Actinomycetales
          FAMILY—Actinomycetaceae
            GENUS—Actinomyces
            GENUS—Nocardia
          FAMILY—Streptomycetaceae
            GENUS—Streptomyces

Figure 21. Classification of systemic monomorphic molds.

in cattle that is commonly referred to as "lumpy jaw." Cervico-facial actinomycosis is the most commonly occurring form of the disease in humans. Another type of infection, thoracic actinomycosis, is caused by aspiration of the organism from the mouth. Eventually, draining sinuses may penetrate to the body surface and the purulent exudate contains the typical "sulfur granules."

8-6. Abdominal actinomycosis may develop from traumatic injuries to the intestinal wall, since *Actinomyces israelii* is often present asymptomatically in the human intestine. Occasionally, it develops in the appendiceal area with no evidence of external trauma. This disease may occur anywhere in the body through blood stream dissemination; however, this is the exception rather than the rule.

8-7. **Laboratory Diagnosis of Actinomyocosis.** *A. israelii* and *A. bovis* have the following general physiological characteristics. They both require anaerobic or microaerophilic conditions and an enriched medium such as brain-heart-infusion agar, brain-heart-infusion blood agar or thioglycollate broth for primary isolation. The optimum pH range of the media is between 6.8 and 7.4 with incubation at 37° C. Frequent transfers are necessary to maintain these organisms, since they tend to die off quite rapidly due to the depletion of essential growth factors and the accumulation of acids.

8-8. See table 20, The Physiological Characteristics of *Actinomyces israelii, A. bovis, A. naeslundii* and Anaerobic Diphtheroids. From a morphological standpoint most strains of *A. israelii* produce rough (R) colonies while the majority of *A. bovis* isolates are smooth (S). The (R) colonies of *A. israelii* are dull white, heaped up, and irregular and show spiderlike filaments on the agar surface. Mature colonies give the appearance of a molar tooth. In thioglycollate broth *A. israelii* produces discrete, lobated, breadcrumb-type colonies about 1 centimeter below the surface. Gram stain of this growth reveals branching filaments which readily fragment to coccoid, bacillary or dipther-

TABLE 20

PHYSIOLOGICAL CHARACTERISTICS OF ACTINOMYCES ISRAELII A. BOVIS, A. NAESLUNDII, AND ANAEROBIC DIPHTHERIODS

	Actinomyces israelii	*Actinomyces bovis*	*Actinomyces naeslundii*	*Anaerobic diphtheroids*
Maximum Growth Reached	3-7 Days	2-3 Days	1-2 Days	3-4 Days
O_2 Requirements	Anaerobic (or microaerophilic after first isolation)	Anaerobic (or microaerophilic after first isolation)	Facultative, especially in presence of increased CO_2.	Anaerobic or microaerophilic
Aerobic conditions	0 or 1^+	0 or 1^+	1^+ to 2^+	0 or 1^+
Air + 10% CO_2 (Candle jar)	0 or 1^+	0 or 1^+	4^+	0 or 1^+
Anaerobic conditions (N_2 + CO_2)	4^+	4^+	4^+	4^+
Catalase production	0	0	0	+
Starch hydrolysis	± or 0	4^+	± or 0	0
Nitrate reduction	80% +	0	90% +	Usually +*
Indol formation	0	0	0	Usually +*
Gelatin liquifection	0	0	0	+ (May take 1-3 weeks)**
Litmus milk reactions	0 to 1^+. acid. No coagulation. Slight reduction. No peptonization	0 to 1^+ acid. No coagulation. Slight reduction. No peptonization	0 to 1^+ acid with slight reduction. Or acid and acid clot. No peptonization	Acid followed by peptonization. (May take 1 to 3 weeks.)**
Sugar Fermentation (Production of acid only) Glucose	+	+	+	+
Xylose	80% +	0	0	0
Mannitol	80% +	0	0	+
Raffinose	Variable	0	80% +	Variable
Starch	20% +	+	20% +	0
Glycerol	0	Occasionally +	0	+

* With *Corynebacterium acnes*, usually either nitrite or indole, or both are produced.

** Several non-proteolytic species of anaerobic *Corynebacterium* have been reported, but these appear to be rare.

456

TABLE 21

MORPHOLOGICAL CHARACTERISTICS OF ACTINOMYCES ISRAELII, A. BOVIS, A. NAESLUNDII, AND ANAEROBIC DIPHTHERIODS

Morphology	*Actinomyces israelii*	*Actinomyces bovis*	*Actinomyces naeslundii*	*Anaerobic Diphtheroids*
A. Gross Morphology on BHI Agar with Anaerobic incubation at 37° C. 1. Colonies examined after 48 hours under the microscope 100X.	Colonies usually only seen microscopically. They appear as a loose mass of long branching filaments on agar surface ("spider" colonies). Or, appear as small whitish granules with a rough surface and a fringed "lace-like" border. Smooth surfaced pin-head sized colonies with slightly fuzzy edges may occur in some strains—"S" forms.	Colonies are usually pinhead in size, transparent, and look like dew drops. They appear smooth, slightly convex with entire edge. Microscopically they show a smooth but granular surface with a granular or denticulate edge. Some strains are more opaque, rough surfaced and have an irregular or fuzzy border. Rare strains are microscopic in size, and appear as mycelial "spider" colonies as seen in "R" A. *israelii* strains.	Colonies similar to those of A. *bovis* or A. *israelii*. "S" forms most common.	Pin-head sized smooth, transparent glistening colonies with smooth edge.
2. Colonies examined after 7 to 10 days.	Raised irregular to lobulated colonies with white glistening surfaces ("molar tooth" colonies). They tend to indent the agar and are easily moved as a whole. Smooth surfaced colonies which are slightly convex with smooth edges may occur in some strains—"S" forms.	Colonies smooth convex, cream to white and shining with entire border. Some strains show conical or irregular lumpy surface and scalloped borders (may look like an inverted raspberry). Rare strains produce typical "molar tooth" colony seen in "R" A. *israelii* strains.	Colonies similar to those of A. *bovis* or A. *israelii*. "S" forms most common.	Smooth colonies which may show granular surface, and entire, slightly granular, edge.
B. Growth in Thioglycollate Broth at 37° C.	Distinct colonies which are rough and lobulated or show fuzzy edges. Colonies do not break up when tube is shaken. Broth is clear. Smooth strains may appear more diffuse.	Most strains produce a soft, diffuse growth. Other strains produce large lobulated breadcrumb colonies which are easily broken up. Flaky or mucoid growth is seen in some strains. Rare strains produce granular discrete colonies as seen in "R" A. *israelii* strains.	Rapid growth—usually more diffuse than A. *bovis*, granular or floccose colonies may be present. Broth somewhat cloudy.	Rapid growth—diffuse, and often pink colored. Tends to concentrate along side of tube. Colonies easily broken up. Broth cloudy.
C. Microscopic Morphology.	Gram positive rods and branched forms, 1 micron or less in diameter. Variations in diameter and clubbed ends are common. Long mycelial filaments occasionally seen. Nonbranching diptheoroid-like rods only may be formed by "S" forms.	Gram positive diphtheroid forms most common. Difficult to find branching. Some strains somewhat more filamentous. Rare "R" strains show long branching filaments.	Similar to A. *bovis* or A. *israelii*, but more irregular forms. Gram positive short mycelial forms with many branches. Some thick, very irregular forms, and few long mycelial elements which vary in thickness throughout. Some diphtheroid-like forms.	Gram positive bacillar or slightly branched organisms. "X" or "Y" shaped forms commonly occur.

480

iods forms. The (S) colonies of *A. bovis* are lacking in spidery filaments, and appear moist, compact, rounded, and glistening. When cultured in thioglycollate broth, a turbid appearance is common, with many of the organisms settling to the bottom of the tube. Several types of growth patterns are produced by *A. bovis* in thioglycollate broth, depending on the strain. A Gram stain of this (S) type growth will usually reveal only bacillary forms due to the fragility of the filaments.

8-9. The clinical specimen usually received in the mycology laboratory is pus, either aspirated from a closed lesion or collected in a sterile tube from a draining sinus. Sputum may be submitted in those cases where pulmonary actinomycosis is suspected. The pus or sputum should be spread out and searched carefully for "sulfur granules." The tiny granules, 2 to 5 mm. in diameter, are round and firm in consistency. They should be removed and placed in a small amount of sterile saline prior to preparation of fresh mounts, stained smears and cultures. If the granules are gently pressed between slide and coverslip for direct examination, we see a lobated, opaque body under low magnification. There may be gelatinous projections, or clubs, over the surface of the granule; however, these sometimes do not occur. High magnification shows the granule to be composed of intertwined, delicate, and branched filaments of 1 micron or less in diameter.

8-10. The granule should be crushed and smeared out on the slide for Gram and acid-fast staining. The Gram-stained slide will reveal thin Gram-positive, branched mycelium and many short lengths of Gram-negative mycelium which closely resemble bacilli. These Gram-negative, rod-shaped forms are the result of physiological changes in older colonies. If no granules are found in the pus or sputum, smears of the clinical material should be stained and examined for Gram-positive branched forms. If non-acid-fast, branching, Gram-positive, thin forms resembling *Actinomycetes spp.* are observed, or if the physician suspects Actinomycosis on the basis of the clinical picture, cultures should be made.

8-11. Suspicious colonies can be picked from streaked plates and inoculated in thioglycollate broth which will more readily reveal the typical branching forms. Pure cultures are essential for use in physiological testing. *Actinomyces naeslundii*, a common inhabitant of the mouth, has to date demonstrated no pathogenic properties and may be readily differentiated from *A. israelii* or *A. bovis* by its ability to grow aerobically. Anaerobic diphtheriods which closely resemble the (S) forms of *A. bovis* and *A. israelii* fortunately can be read-

TABLE 22

LABORATORY IDENTIFICATION AND DIFFERENTIATION OF PATHOGENIC NOCARDIA AND STREPTOMYCES

	Nocardia asteroides	*Nocardia brasiliensis*	*Streptomyces sp.*
Demonstration of Branched Mycelium, characteristic of *Actinomycetes* (slide culture)	Branched mycelium form	Branched mycelium form	Branched mycelium form
Demonstration of acid-fast staining	Partially acid-fast	Partially acid-fast	Not acid-fast with exception of occasional spores
	Other species of *Nocardia* are not acid-fast		
Hydrolysis of Casein	Does *not* hydrolyze casein	Readily hydrolyzes casein	Readily hydrolyzes casein
Growth in Gelatin	Does not grow; or grows poorly producing thin, flaky, white growth	Readily grows, producing compact, round colonies	May or may not produce good growth; growth will be stringy or flaky
Pathogenicity to guinea pig	Usually pathogenic to guinea pig	Usually *not* pathogenic to guinea pig	Not pathogenic to guinea pig

458

TABLE 23

AEROBIC AGENTS OF ACTINOMYCOTIC MYCETOMA—GEOGRAPHIC DISTRIBUTION AND APPEARANCE IN TISSUES

Species	*Geographic Distribution*	*Appearance in Tissues*
Streptomyces somaliensis	Common cause of mycetomas in Africa Also present in Saudi Arabia, Brazil, and Mexico	Grains usually present. They are large (up to 1.25 mm D.), yellowish to slightly pink, round or oval, compact and very hard. Microscopically, grains consist of a matrix of amorphous material with mycelial filaments abundant near the periphery, but not at edge of granule. No clubs have been observed.
Streptomyces pelletierii	Cause of mycetomas in Africa and Latin America	Grains usually present. They are small (less than 1 mm D.), deep garnet red with irregular shape and smooth or denticulate edges - often lobulated. Microscopically, the grains show a homogenous matrix, and mycelial filaments are difficult to see. No clubs have been observed.
Streptomyces madurae	Cause of mycetomas all over the world. Reported from: North, Central, and South America, Africa, India, Europe, and Australia	Grains usually present. They are very large (more than 1 mm D.), yellowish to white or slightly pink, usually irregular to serpiginous in shape. Microscopically, center of grain appears empty or only loosely filled with mycelial filaments. A dense network or mantle of filaments surrounds central area. These filaments radiate outward and are surrounded by amorphous material forming clubs. Clubs are usually numerous and very long (up to 25 microns) may taper to pointed ends or may branch.
Nocardia brasiliensis	Common cause of both mycetomas and pulmonary disease in Mexico. Also present in Central America, South America, Africa and the United States, and India	Grains usually present. They are small, yellowish, lobulated and soft. Microscopically, the grains appear homogeneous. Mycelial filaments are difficult to observe. Clubs may or may not be present. If grains do not occur, organism is seen as freely branching filaments (acid-fast) in the tissues.
Nocardia asteroides	Cause of both systemic disease and mycetomas all over the world	Grains may or may not occur. They are very small, cream to white. Microscopically, the grains are homogeneous or composed of a loose clump of mycelial filaments. Clubs may or may not be present. If grains do not occur, organism is seen as freely branching filaments (acid-fast) in the tissues.

ily differentiated on the basis of biochemical tests. Refer to tables 20 and 21 for the morphological and physiological characteristics of the actinomyces and the anaerobic diphtheroids.

8-12. **Nocardiosis.** *Nocardia spp.* cause a chronic or acute granulomatous or suppurative disease in man and animals which may be systemic, resembling tuberculosis, or subcutaneous in the form of a mycetoma. Several filamentous members of the order *Actinomycetales* produce human disease in addition to *Actinomyces israelii.* At one time, they were all classed in the *Genus Nocardia.* Some of them are now considered to fall in the Genus *Streptomyces,* and they also will be discussed in this section. The causative fungi of Nocardiosis are saprophytic in the soil, and therefore, the disease is exogenous in origin. The disease is cosmopolitan in distribution with greater prevalence in the tropic and subtropical areas of the world. Pulmonary infections result from the inhalation of the causative fungal agent, while the mycetomal form normally results from the penetration of the fungus due to injury. No transmission between individuals or animals and humans takes place. Refer to table 22 for species differentiation.

8-13. *Clinical aspects of Nocardiosis.* The pulmonary disease is caused primarily by *Nocardia asteriodes. Nocardia brasiliensis* may also cause this form of the disease, but the organism is more often associated with the mycetomal type of infection. As previously stated, pulmonary nocardiosis occurs as a result of inhalation of the fungal spores, and the infection often remains confined to the lungs. Blood stream dissemination is not unusual and the organism attacks the central nervous system in about a third of the cases of disseminated nocardiosis. After entry to the lungs, single lesions may develop, but the more common picture is one of scattered infiltration of a military type resembling tuberculosis. There is a tendency for the lesions to penetrate the pleural wall, causing abscesses on the chest wall and rib involvement. As a

482

TABLE 24

AEROBIC AGENTS OF ACTINOMYCOTIC MYCETOMA CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS

SPECIES	MORPHOLOGY IN CULTURE (Sabouraud Dextrose Agar)	PHYSIOLOGICAL CHARACTERISTICS			
		Optimum Temperature	*Proteolytic Activity*	*Amylolytic Activity*	*Pathogenicity for guinea pig or mouse*
Streptomyces somaliensis	Colony (Gross): Creamy colored wrinkled flaky growth loosely adherent to agar. May develop a tan aerial mycelium. Old colony may be brown or blackish. No diffusible pigment. Microscopic exam: Delicate branched mycelium less than 1 micron in diameter. Aerial mycelium may show chains of spores. Mycelium not acid-fast.	30°	+	+	Pathogenicity tests in mice and guinea pigs not successful
Streptomyces pelletierii	Colony (Gross): Very small glabrous, dry granular adherent colonies. Fungus grows very slowly. Colonies first pale pink, then deep garnet red. No aerial mycelium. No diffusible pigment. Microscopic exam: Delicate branched mycelium, less than 1 micron in diameter. Conidia rarely seen. Not acid-fast.	37°	+	0	Pathogenicity tests in mice and guinea pigs not successful
Streptomyces madurae	Colony (Gross): Moderately fast growing adherent cream colored glabrous colony with a firm hard surface usually shiny and wrinkled. Colonies may develop a whitish powdery aerial mycelium. Microscopic exam: Delicate branched mycelium, less than 1 micron in diameter. Chains of conidia may be seen. Not acid-fast.	37°	+	+	Pathogenicity tests in mice and guinea pigs not successful
Nocardia brasiliensis	Colony (Gross): Rapid growing, heaped wrinkled or folded colony. Pale tan to yellowish, orange or red-orange. May be glabrous or covered with white powdery aerial mycelium. Some strains produce browning of the medium. Microscopic exam: Short irregular rods and occus forms predominate. Long branched mycelial forms best seen in liquid or slide culture preparations. Forms less than 1 micron in diameter, partially acid-fast, often beaded. Terminal conidia seen in some strains.	30° (Some strains grow well at 37° also.)	+	0	Abscesses and grains may be produced in mice and guinea pigs with some strains
Nocardia asteroides	Same as above.	37° (Some strains grow better at 30°.)	0	0	Usually more virulent for the guinea pig than *N. brasiliensis*. Abscesses without grains. Frequently causes death

result of these extensions, thoracic skin lesions of a draining sinus type may develop.

8-14. Actinomycotic mycetoma may result from injuries contaminated with *Nocardia brasiliensis, Streptomyces madurae, Streptomyces pelletieri,* or *Streptomyces somaliensis.* The gross morphology of two of these fungi is shown in detail B of foldout 2. Frequently the foot is involved, since many people go barefoot in the warm humid areas of the world. The fungal tumor resembles that described for mycetomas due to *Actinomyces israelii.* The variations result because the different etiological agents of mycetoma may have an affinity for different tissues. Also, "sulfur granules," when they are produced, will vary considerably in morphology and color, dependent on the causative fungal agent. Refer to table 23 for geographic distribution and appearance in tissue.

8-15. *Laboratory diagnosis of nocardiosis.* Clinical materials such as pus, sputum, tissue or spinal fluid are collected in sterile containers. Pus and sputum should be carefully examined for grains, although they are generally absent from the sputum in cases of pulmonary nocardiosis. Acid-fast and Gram stains should be performed on fresh materials, such as centrifuged sediment in the case of spinal fluid or concentrates of sputum and gastric washings. When grains are found, they should be grossly examined and then crushed and stained and microscopically examined. The presence of partially acid-fast, Gram-positive, thin-branched mycelium is indicative of a Nocardial infection. Refer to table 23 for a resume of the gross and microscopic appearance of grains from tissue.

8-16. The specimen should be cultured on Sabouraud's dextrose agar without antibiotics, since *N. asteriodes* and *N. brasiliensis* are sensitive to these substances. It has been demonstrated that some strains of these organisms lose their viability when subjected to the digestion and concentration techniques normally employed for the recovery of tubercle bacilli; therefore, the specimen should be inoculated on culture media both prior to and following concentration. Multiple cultures should be inoculated to permit incubation at both room temperature and at 37° C. This is necessary because some strains of *N. asteriodes* will grow well at one temperature and not at the other. The technician should be aware of the fact that these organisms grow quite readily on media used for *M. tuberculosis,* particularly if incubated at 37° C. The colonies of *Nocardia spp.* will develop earlier than the tubercle organism (within 1 to 2 weeks) and appear similar to the "atypical" or saprophytic forms of mycobacteria. In order to detect the partially acid-fast branched mycelium of *Nocardia spp.* a careful examination of the early growth is necessary and may be quite rewarding.

8-17. Animal inoculation has been shown to be impractical for routine diagnosis. Identification should be established on the basis of morphological and physiological characteristics. The biochemical tests routinely used for identification and differentiation of the *Nocardia spp.* and the *Streptomyces spp.* include the casein hydrolysis test and the gelatin test. Study table 24 for the laboratory identification and differentiation of pathogenic *Nocardia* and *Streptomyces spp.*

The Pathogenic Dimorphic Fungi

THE TERM "dimorphic," which implies two shapes, refers to that group of fungi that have the unique capability of existing in two morphologically distinct forms. When incubated on an artificial substrate at 25° C., these organisms will grow in a moldlike filamentous form; incubation of the same organism on the same substrate at 37° C. will result in the development of mucoid, bacterialike colonies of budding yeast cells.

2. The filamentous moldlike growth occurs in nature and is referred to as the *saprophytic* form. Human and animal infections result when spores produced by the saprophytic form are introduced into the body either from tissue damage or by inhalation. Following entry, the yeast or parasitic form of the fungi develops in the host, whose temperature approximates 37° C.

3. The dimorphic, or as they are sometimes called, the diphasic fungi, cause the most serious fungal diseases in man. This chapter will discuss *Sporotrichum schenkii, Coccidioides immitis, Blastomyces dermatitidis, Paracoccidioides brasiliensis* and *Histoplasma capsulatum* from the standpoint of their clinical effects in man and the methods used in laboratory identification.

9. Subcutaneous Group

9-1. With the exception of *Sporotrichum schenkii,* which generally infects subcutaneous tissues, the dimorphic fungi cause diseases that are incurable unless diagnosed early so that proper therapeutic treatment can begin. They also confuse diagnosis. It has been estimated that about 8 percent of those people confined to tubercular sanitariums actually are infected with chronic pulmonary histoplasmosis, a disease caused by *Histoplasma capsulatum.* Patients ill with histoplasmosis are also likely to contract tuberculosis. On the other hand those people with tuberculosis are not subject to histoplasmosis from other patients because the disease is not transmissible in the parasitic or yeast form. This principle holds for all the pathogenic diphasic fungi.

9-2. When working with the dimorphic fungi in pure culture, a bacterial safety hood is essential. Extreme caution and the best possible aseptic technique must be used continually to prevent laboratory infections. The highly infectious spores produced by the mold form of these fungi easily become airborne if the culture tube is shaken or handled roughly.

9-3. Sporotrichosis, caused by *Sporotrichum schenkii,* is usually a chronic, subcutaneous, lymphatic mycosis and is found worldwide. On rare occasions this fungal agent may disseminate to cause generalized infection of the bones, joints, and body organs. *Sporotrichum schenkii* has a wide distribution in nature. Investigators have found it as a saprophyte on living and dead vegetative matter as well as in animal excreta. One particular investigation demonstrated that its growth on mine timbers was markedly enhanced if the temperature was maintained between 79° F. and 84° F. and the relative humidity never fell below 92 percent. The disease is found predominantly in people who work and play outdoors. Florists are frequently exposed to infection by virtue of handling thorny plants which may be harboring the organism.

9-4. Localized lymphatic sporotrichosis, the most common form of the disease, normally results from implantation of the spores in the subcutaneous tissue during a penetrating injury from a thorn or splinter. In spite of topical treatment, the small ulcerated lesion which develops will not heal; it develops into a firm subcutaneous nodule, eventually adhering to the skin. The color of the lesion changes from pink to black as the infection ruptures through the skin to form the typical necrotic chancre. The course of the disease is characterized by a chainlike lymphatic involvement, with swelling of nodes and induration of the connecting lymphatics. The primary lesion as well as the necrotic nodes in closet proximity to it have a tendency to ulcerate and excrete a purulent drainage.

9-5. Disseminated sporotrichosis is uncommon, probably due to an immune response in those indi-

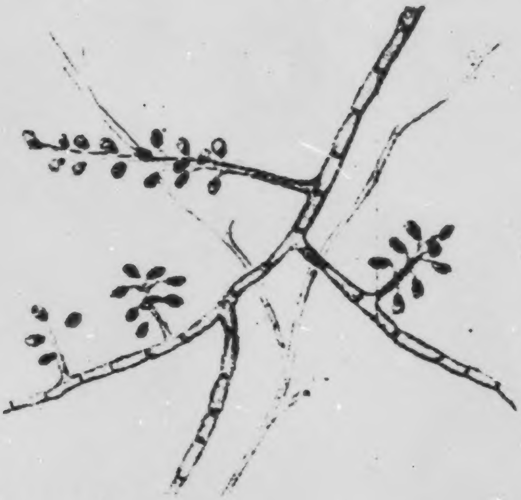


Figure 22. Sporotrichum Schenkii (mycelial phase).

viduals suffering from the chronic lymphatic form of the disease. In a few cases, sporotrichosis may be spread throughout the body, either via the bloodstream or as a result of suppurating lymph nodes. Manifestations of disseminated sporotrichosis include the development of numerous and widespread cutaneous lesions. Oral and nasal mucosa involvement is quite common. Lesions may be found in the kidneys, lungs, bones, joints, muscles, genitourinary system, and other body organs.

9-6. A direct examination of clinical material, i.e., scrapings from skin lesions or swabs from draining ulcers, is generally of little or no value. In Gram-stained smears, it is very difficult to distinguish the few organisms which may be present from other tissue elements. Regardless of the findings by direct examination, cultures should be inoculated to Sabouraud's dextrose agar containing the antibiotics cycloheximide and chloramphenicol. In order to minimize bacterial contamination of clinical material it is best to use pus aseptically aspirated from an unruptured nodule. The mycelial phase of *Sporotrichum schenkii* grows rapidly at room temperature. Within 3 to 7 days, moist white colonies appear; they soon develop the irregularly wrinkled or folded surfaces seen in detail A-2 of foldout 1. The colony may remain moist or membranous, or its margins may develop a fine greyish velvety appearance. Macroscopic examination after 1 to 2 weeks growth of the fungus reveals a brownish colony which gradually turns black and imparts a yellowish pigment to the medium. There is considerable variation in colony pigmentation in different strains of *S. schenkii* or even in the same strain. This is due to variations in oxygen supply and the amount of thiamine in the medium.

9-7. Microscopically, in teased wet mount a fine (2 microns in diameter), branching, septate mycelium with pyriform, oval, or spherical conidia on delicate sterigmata is seen. The conidia occur in two distinct patterns shown in figure 22. One possible pattern is the so-called "sleevelike" arrangement where the spores are borne individually on delicate sterigmata along the length of a hyphal filament. A second frequently occurring pattern, termed a "flowerette" arrangement, forms if each spore is attached by its own delicate sterigma to a common conidiophore. For best observation of these patterns, the slide culture technique is recommended.

9-8. The tissue, or parasitic, phase of *Sporotrichum schenkii* bears little similarity to the mycelial phase described above. The organism seen in tissue, or when cultured under 5-percent CO_2 at 37° C. on enriched media such as brain-heart-infusion agar, appears in the form of creamy white colonies of yeastlike budding cells, which due to their elongated shape are called "cigar bodies." The "cigar bodies" are difficult to find using ordinary stains on human tissue. Methenamine silver stains and fluorescent antibody techniques seems to delineate these organisms quite clearly from surrounding tissues. The macroscopic and microscopic appearance of the yeast phase are shown in figure 23 and detail A, of foldout 1.

9-9. A confirmatory diagnosis of *S. schenkii* requires demonstration of the yeastlike phase of the organism. This may be accomplished by culturing the mycelial phase on brain-heart-infusion agar, with or without blood, under CO_2 at 37° C. The surface of the medium should be moist. It is often

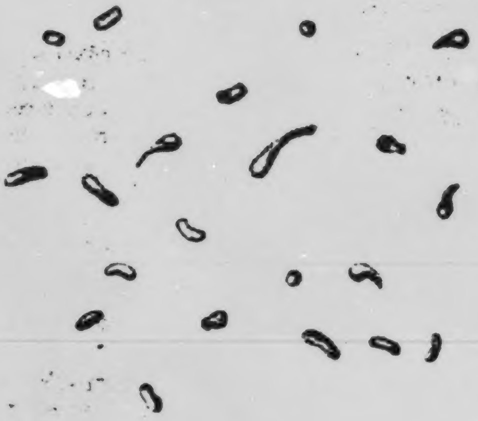


Figure 23. Tissue phase of Sporotrichum schenkii.

possible to discern microscopic areas of conversion from the edges of the mycelial inoculum before grossly visible yeastlike growth emerges. If conversion does not take place on artificial media, animal inoculation may be accomplished. Use several mice inoculated intratesticularly with 0.2 ml. of a dense mycelial suspension. After the first week, and if necessary at additional intervals, pus may be removed from the testes and examined by Gram stain for the typical oval or elongated budding cells of the parasitic or yeast form of *S. schenkii*. Serological tests for this disease have not yet been standardized. Recent studies indicate that fluorescent antibody techniques can be successfully adapted for use in the serologic diagnosis of Sporotrichosis from clinical specimens as well as from culture.

10. Systemic Group

10-1. These pathogenic diphasic fungi cause deep-seated diseases which involve one or more of the internal organs of the body. In many instances the bones and subcutaneous tissues are also infected, and frequently as a result of bloodstream dissemination skin lesions may be manifested. Immunologic tests and skin tests demonstrating past or present infection with some members of this group indicate that many individuals have had subclinical or asymptomatic exposure which otherwise might have gone undetected. In some asymptomatic individuals an indication of systemic mycotic lesions may be seen in X-ray examination, but confirmation of diagnosis may not be reached unless an autopsy is performed after death and the organism identified. Most often the portal of entry for the infective spores of these organisms is the respiratory system and early symptoms may be nondiscrete. If the disease goes undiagnosed and improperly treated, the likelihood of its becoming progressive is enhanced, and such cases frequently prove fatal.

10-2. **Coccidioides Immitis.** This fungal agent is responsible for the systemic disease—coccidioidmycosis. The disease is geographically limited to specific areas of the New World, and since one of these areas happens to be the San Joaquin Valley in south central California, the infection has become known as "valley fever." *C. immitis* exists as a saprophyte in the soil, producing innumerable infective spores which are readily borne by air currents over appreciable distances. Areas other than California which provide suitable climatic conditions for its propagation include Arizona, New Mexico, Texas, and the northern portion of Mexico. Males between 30 and 50 years of age and the darker skinned races are particularly susceptible to infection. Experience has shown that preventive measures to control exposure to dust, such as wetting down dusty areas, planting lawns, and even the wearing of protective masks, markedly reduces the incidence rate in endemic regions.

10-3. Primary pulmonary coccidioidomycosis is contracted by inhalation of spores; more than half of those so infected remain asymptomatic and develop an immunity to the disease. Others develop mild symptoms resembling those of the common cold. Some individuals develop severe pulmonary symptoms. As many as 30 percent of the severely infected patients develop allergic skin eruptions in response to toxins produced by this organism. Approximately 999 of 1000 cases gain complete recovery. These people continue to demonstrate a positive skin test with coccidioidin for many years, and are seemingly imparted with a lifetime immunity to the disease.

10-4. Another form of this disease, disseminated coccidioidomycosis, fortunately occurs only in rare instances and probably is directly related to some defect in the patient's immune response. This form of the disease may progress in several patterns with regard to time and extent of involvement. The organism may be spread throughout the body by the bloodstream early in the respiratory phase, resulting in massive involvement. In other cases individuals who are unaware that the disease is present suddenly manifest a single cutaneous lesion that may worsen or, on occasion, heal spontaneously. The body sites most commonly infected following dissemination from the primary lung lesion include other areas of the lungs, subcutaneous and cutaneous tissues, bones, joints, meninges, the brain, and the viscera. Cutaneous dissemination typically results in the formation of subcutaneous abscesses.

10-5. Clinical materials such as pus, sputum, pleural fluid, or bronchial washings should first be examined in direct wet, unstained mount for the presence of spherules demonstrated in figure 24. These spherules, when mature, have thick refractile walls and contain numerous endospores. If distinction between fungus and granulocytic cells is difficult, the preparation may be sealed and allowed to stand for several hours, or as long as overnight. If the observed structures are actually spherules, the undischarged endospores will have developed mycelial filaments.

10-6. Whether or not spherules are identified in direct wet preparation, the clinical material should be cultured at room temperature on a selective medium such as Sabouraud's dextrose agar with antibiotics. It is best to use cotton-plugged tubes since the microaerophilic condition produced in sealed tubes is inhibitory to sporulation in some strains of *C. immitis*. After the third to fifth day the colonies that appear will be moist, flat and

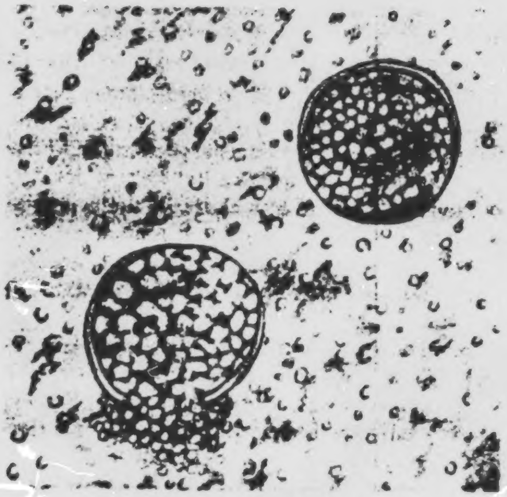


Figure 24. Spherules of coccidioides immitis.

grey. Gradually, an abundant white, fluffy, aerial mycelium develops over the mature colony as we find in foldout 2, detail C. The pigment on the underside of the colony in most strains is a brownish-black. This imparts a greyish appearance to the growth. Typically, some areas of aerial mycelium become flattened, causing a glabrous effect.

10-7. The mature colony is now examined microscopically in teased wet mount preparation for the presence of barrel-shaped, tagged arthrospores seen in figure 25. Characteristically, these arthrospores develop in alternating segments of the hyphae so that a single small undifferentiated cell separates the arthrospores from each other. When mature, these chains separate very readily, and arthrospores carry with them fragments of the small dividing cells, thus initiating the descriptive term "tagged" arthrospore. Investigations have demonstrated the presence of spherules in mycelial cultures. By using special cultural methods not readily adaptable to diagnostic work, they have partially converted the mycelial phase to the spherule or tissue phase. In such experimental cultures, you can see arthrospores gradually developing into spherules, which on reaching maturity send out germ tubes through the spherule wall to form a new mycelium. Extreme care must be exercised in handling tubes of mycelial growth. Prior to removal of the cotton plug, the growth should be flooded with sterile normal saline by inserting a large-gauge syringe needle into the tube alongside the plug. This retards aerosol formation by arthrospores. The inability of saprophytes resembling *C. immitis* to grow on media containing cycloheximide is a characteristic very useful in screening suspicious growth.

10-8. In order to establish a confirmatory diagnosis of coccidioidomycosis, 0.1 ml. of suspension of mycelial growth is inoculated intratesticularly in a guinea pig. Orchitis will normally develop within a week, in which case fluid is aseptically withdrawn and examined microscopically in wet preparations for the presence of spherules. When guinea pigs are not available, mice may be substituted; however, the injection should be made intraperitoneally using 1.0 ml. of inoculum. The lesion or lymphatic exudate is examined as above, following the death of the mice. The finding of spherules containing endospores is confirmatory.

10-9. Several immunological procedures such as the precipitin test and the complement-fixation test have proven very useful in diagnosis of coccidioidomycosis. The coccidioidin skin test is an extremely useful tool for epidemiological studies, however, it is of limited diagnostic value since a positive reaction may indicate either a fully recovered or a presently active case. The precipitin test usually becomes positive before there is a demonstrable rise in the complement-fixation titer. The precipitins (antibodies) appear within the third to fifth day after onset and reach a maximum level in 1 or 2 weeks. The complement-fixation test is only useful in moderate to severe cases of the disease. Those patients with primary coccidioidomycosis maintain a moderately low titer for several months, which gradually falls as the patient recovers. When the disease is progressive, the titer rises sharply and remains there until the patient's condition improves. Improvement causes the titer to gradually diminish.

10-10. **Blastomyces Dermatitidis.** North American blastomycosis, Gilchrist's disease, or simply blastomycosis, are synonyms for a disease caused

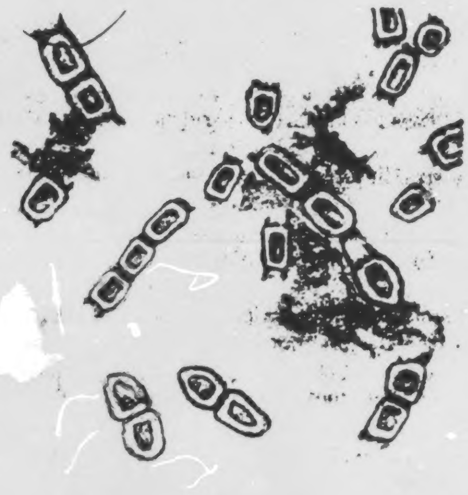


Figure 25. Arthrospores of immitis.

by the diphasic fungus, *Blastomyces detmatitidis*. The disease is chronic, characteristically causing both suppurative and granulomatous lesions that normally originate in the respiratory system. The infection is particularly well-named, since only a few confirmed cases have been diagnosed outside North America. The organism seems to be even further limited in distribution to the northern part of the Mississippi Valley, the Ohio River Valley and the Middle Atlantic states. For many years there was considerable doubt as to the portal of entry of *B. dermatitidis* into the body; however, in recent years investigators have demonstrated this fungus in the soil and now believe that the majority of infections are acquired by inhalation of infective spores.

10-11. The clinical forms of the disease are sometimes separated into four categories: primary cutaneous, primary pulmonary, chronic cutaneous, and disseminated. The latter three will be discussed as a single entity since they are so closely related. The primary cutaneous infection is extremely rare and results from accidental inoculation of the organism through the skin. A papule appears at the site of entry. Gradually, a chain of swollen lymph nodes and vessels develops. The organism remains localized in the infected part, much the same as with sporotrichosis.

10-12. Primary pulmonary blastomycosis, based on present evidence, is most probably the precursor of both the disseminated and chronic cutaneous forms of the disease. In its early phases, symptoms usually are indistinguishable from those of the common cold. When dissemination occurs, the tissues most commonly involved are the skin, the bones, especially the vertebrae and ribs, and the viscera. About one-third of the cases manifest lesions of the central nervous system. What is now believed to be a special type of dissemination from pulmonary origin to the skin results in the chronic cutaneous form of this disease. Other tissues of the body remain uninvolved. This most commonly occurring form of the disease may last for years. It characteristically starts as an isolated papular lesion or subcutaneous nodule which becomes an abscess and then ruptures to form an ulcer. New lesions may result from the pulmonary focus or by implantation of the organism to new skin areas from older draining ulcers. It has been estimated that as much as one-sixth of the body surface may become ulcerated with this form of disseminated blastomycosis.

10-13. Direct examination of clinical materials often proves quite fruitful in cases of North American blastomycosis. In many instances the technician is able to demonstrate the etiological agent in large numbers and thereby lay the groundwork for a provisional diagnosis. Pus or scrapings should be obtained from the active areas of cutaneous lesions. When primary pulmonary blastomycosis is suspected, sputum or bronchial washings may be examined. When specimens such as urine, gastric washings, spinal fluid or pleural exudates are collected for laboratory examination the physician undoubtedly suspects the dissemination of the organism. These clinical materials should be examined microscopically in unstained preparations. Staining, e.g., the Gram-stain, makes it most difficult to distinguish these organisms from lymphocytes. Whenever the volume of specimen permits, the sediment obtained from centrifugation should be studied. If the specimen is dry or dense, as may result with skin scrapings, a drop of water may be added prior to coverslipping the material. Regardless of what form of clinical material is examined, the technician must look for the characteristic budding cells produced by the tissue phase of this fungus. These cells are shown in figure 26. In the event of negative findings, the coverslip may be sealed and the specimen incubated for several hours at room temperature. If cells of *B. dermatitidis* are present, they will typically put out single germ tubes as opposed to the multiple germ tubes put out by the spherules of *C. immitis*.

10-14. Cultural studies should be used regardless of the findings on direct examination. The clinical material is inoculated to an enriched medium such as brain-heart-infusion agar (in addition to Sabouraud's dextrose agar) since some strains have been shown to possess fastidious nutritional requirements. If cycloheximide is added to the medium, incubation should be made at room temperature as the yeast phase of *B. dermatitidis* is sensitive to this antibiotic at 37° C. The mycelial phase is slow-growing and may require up to 2 weeks to become visible. On Sabouraud's dextrose agar at room temperature growth may at first appear moist or membranous; however, an aerial mycelium forms rapidly, creating a downy to fluffy colony which at first is white, later becoming deep cream to tan. See detail D–1, foldout 2, for the appearance of the colonies. Microscopic study of this mycelial growth in teased wet mount reveals a septate mycelium and nondiscrete conidia, round to pyriform in shape, ranging from 3 to 5 microns in size. These conidia are borne either sessile (affixed directly to the hypha), or on the tips of short lateral hyphal branches. The parasitic or tissue phase of *B. dermatitidis* can be readily obtained "in vitro" by inoculating clinical material to both Sabouraud's dextrose agar and brain-heart-infusion agar and incubating at 37° C. Growth is slow and the colonies are typically cream to tan in color and moist to waxlike in appearance. They are pictured in detail D–2 of fold-

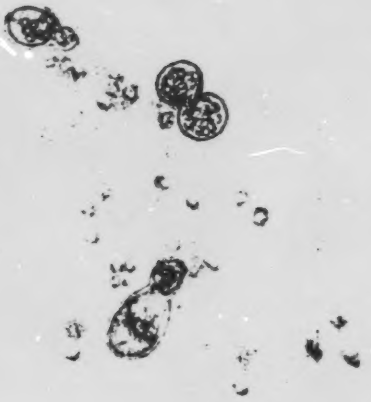


Figure 26. Blastomyces dermatitidis.

out 2. The topography is normally rough; however, some strains have been reported to produce smooth, convex colonies. The microscopic picture is quite similar to that seen in direct examination of clinical material from a patient, or from inoculated animals. Large oval to round cells usually about 8 to 10 microns in diameter, sometimes reaching as much as 20 microns, will be seen. These cells are typically single budding and have a thick, refractile wall which is easily distinguishable from the granular cytoplasm that it incloses. The attachment of mother to daughter cell is characteristically by means of a wide base. It creates a configuration which is frequently spoken of as a "figure eight." A few strains of *B. dermatitidis* have demonstrated the capability for multiple budding, but even in such instances the single budding form predominates.

10-15. In order to establish a confirmatory diagnosis of *B. dermatitidis*, it is essential to obtain the yeast or tissue phase of this organism. This can be readily accomplished by cultural methods, and thus the use of animals is unnecessary. Fresh tubes of brain-heart-infusion agar should be inoculated with mycelial growth suggestive of this fungus and incubated at 37° C. After a few days, conversion usually begins at the margins of the inoculum. When this colony material is microscopically examined, the typical, wide-based, thick-walled, single-budding cells of the yeast phase can be seen. Additional confirmation is relatively simple since this yeastlike colony readily converts to the mycelial phase when the culture tubes are incubated at room temperature. Skin testing with a mycelial filtrate called blastomycin has been reliable for indication of past and present infections. The reagent is not as consistent in results as that used for histoplasmosis or coccidioidomycosis. This disparity may be due to a lower level of superficial sensitivity in the tested individuals or to variation in the components of the blastomycin. As in the case of the advanced disseminated stage of coccidioidomycosis and histoplasmosis, all skin sensitivity may disappear. The complement-fixation test for *B. dermatitidis* is useful despite difficulty in standardizing reagents. The titer generally rises as the disease progresses and disappears with recovery. A high complement-fixation titer and a negative skin test usually indicates the terminal phase of the disease.

10-16. **Paracoccidioides Brasiliensis.** Paracoccidioidomycosis, often referred to as South American blastomycosis, is a chronic granulomatous mycotic disease of the skin, lungs and other internal organs with a special predilection for the mucous membranes. Although geographically limited to northern South American countries, especially Brazil, Mexico and Central America, the disease should not be ignored by workers in other global areas. The infection may remain subclinical for many months in travellers from endemic countries. *P. brasiliensis* has been isolated from the soil, and it is believed to exist in nature as either a saprophyte in the soil or as a plant parasite. The disease, therefore, is sometimes acquired by farm workers who use vegetation to pick their teeth or for chewing purposes. There is also recent evidence that the lungs serve as the primary area of infection as a result of inhaling airborne spores. The clinical characteristics of paracoccidioidomycosis will be discussed in two categories: the skin and mucocutaneous form, and the disseminated form.

10-17. Typically, the primary cutaneous lesion produced by this diphasic fungus appears within the mouth or nose, or at a point within close proximity to these structures. The erythematous mucocutaneous ulcerations which develop spread slowly to many regions of the mouth and have an apparent affinity for the tonsillar region. Frequently, there is an extension of mucosal lesions to the skin, or alternative skin lesions may result from auto-inoculation. Other cutaneous lesions may result from subcutaneous deposit of the fungus as a result of lymphatic or bloodstream spread. Involvement of any body surface is possible, and early diagnosis is important because the lesions simulate those caused by cutaneous leishmaniasis, yaws, and tuberculosis.

10-18. Recent evidence indicates that a high percentage of disseminated cases of *P. brasiliensis* occur with the lungs providing the primary loci of infection. The symptoms closely resemble those of tuberculosis, and in numerous cases both diseases have been diagnosed in the same patient. On some occasions the lower gastrointestinal tract appears to be the primary site of inoculation. In such cases there is frequently dissemination to the spleen, liver, appendix, and ano-rectal region. Regardless of the primary site of infection, the lymphatics which drain the site and eventually the entire lymphatic system becomes involved. In the early phases of the common oral form of the disease the cervical nodes of the neck usually become painfully swollen and penetrate the skin surface with sinus tracts which drain a purulent fluid, rich in fungal structures. Central nervous system lesions and bone involvement may occur, particularly in those patients where the organism is disseminated via the bloodstream.

10-19. Direct microscopic examination for the tissue or yeast phase of *P. brasiliensis* should be accomplished. Clinical materials such as pus or scrappings from lesions, sputum, and other body fluids, or aspirated material from infected lymph nodes may be used for this examination. Whether or not the characteristic budding cells are observed in unstained wet preparations, the material must be cultured by methods and materials recommended for the isolation of *B. dermatitidis*. (See paragraph 10-14.)

10-20. On Sabouraud's dextrose agar at room temperature the mycelial phase of *P. brasiliensis* develops very slowly. The small, heaped colony which forms after 2 or 3 weeks seldom exceeds a diameter of 20 millimeters, even after several months of incubation. The majority of strains form a short, white, velvety aerial mycelium covering the colony surface. This morphology is shown in foldout 2, detail E–1. Others develop white to cream-colored glabrous colonies which typically are folded irregularly. The usual microscopic findings in teased wet mount consist of only the septate mycelium and chlamydospores. Some investigators describe the presence of microconidia borne laterally, either directly on the hyphae, or on short sterigmata. These conidia are typically pyriform in shape, ranging from 2 to 3 microns in length. When clinical materials are cultured on brain-heart-infusion agar at 37° C., the growth which slowly develops is yeastlike in appearance, varying from cream to tan in color. The yeastlike colony is illustrated in E–2 of foldout 2, macroscopically, some strains present a verrucose and waxy surface while others may be smooth and shiny. In contrast to the mycelial phase, microscopic examination of a teased wet mount reveals structures which are quite distinctive. Whether the fungus is studied directly in clinical material or from culture at 37° C., the microscopic morphological picture shown in figure 27 is that of relatively large round cells, ranging between 10 and 60 microns in diameter, and possessing well-defined, refractile cell walls. These yeast or tissue-phase cells of *P. brasiliensis* reproduce typically by multiple budding; however, the single budding displayed by other pathogenic organisms is not infrequent. The daughter cells appear to be "pinched-off" as they mature; and they may develop in two distinct morphological patterns. In the first pattern the buds are quite small and vary only slightly in size; this creates an effect spoken of as the "steering wheel" form. A second pattern results when the buds vary appreciably in size, i.e., as much as 10 microns in length. Since budding occurs on all surfaces of the mother cell, and since the connecting points of only a few buds can be seen in any one plane microscopically, a rather bizarre effect is created. The mother cell seems to be surrounded by unconnected cells; in actuality this is not the case. A chaining effect results frequently when daughter cells continue to bud without becoming detached.

10-21. In order to confirm cultural identification it is essential to convert the yeastlike growth to the mycelial form, or the mycelial type growth to the tissue form, depending on which is recovered from initial inoculation. If transformation from tissue to mycelial form is to be attempted, the yeastlike growth should be subcultured on fresh tubes of Sabouraud's dextrose agar and incubated at room temperature. Should the reverse situation exist, mycelial growth is subcultured on fresh brain-heart-infusion agar and incubated at 37° C. A moist agar surface is highly desirable. Further confirmation is gained by reversing incubation temperatures following the development of adequate growth, and thereby inducing conversion.

10-22. Other diagnostic techniques used in suspected cases of paracoccidioidomycosis include animal inoculation, skin testing and the complement-fixation test. Ordinarily, animal inoculation is unnecessary for either isolation or identification of this fungus; however, the intratesticular injection of guinea pigs can be useful in establishing the pathogenicity of a particular strain. Intradermal skin testing, using a filtrate from broth cultures, called *paracoccidioidin*, has met with some success. Although considerable cross-reactivity with histoplasmin and blastomycin occurs, those individuals with paracoccidioidomycosis show a greater response to paracoccidioidin. The complement-fixation test has proven itself useful for diagnosis and prognosis. Typically, the rise in titer parallels the progress of the disease, and the titer diminishes with remission or recovery.

10-23. **Histoplasma Capsulatum.** This systemic diphasic fungus is responsible for the disease histoplasmosis, an intracellular mycosis which is

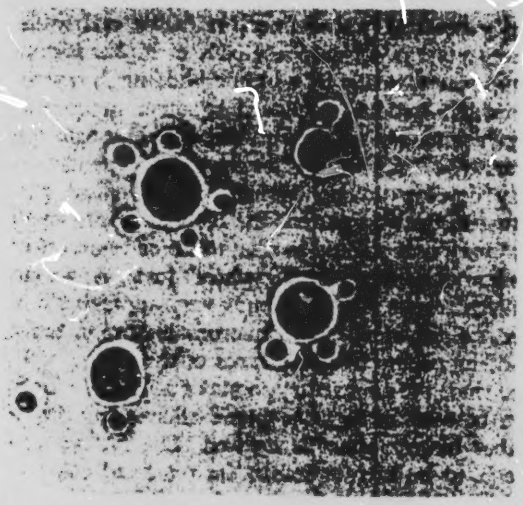


Figure 27. Paracoccidioides brasiliensis.

normally respiratory in origin. The organism demonstrates a distinct preference for the reticulo-endothelial system. The disease at one time was considered rare and invariably fatal; it is now known to be quite common in its benign form, especially in those regions where it is endemic. Although the infection occurs primarily in North America—more specifically in the north central part of the United States and along the eastern seaboard—significant outbreaks have been reported in Central and South America, Australia, South Africa and Europe. *H. capsulatum* exists as a saprophyte in the soil, often in association with the excreta of poultry, bats, and birds, such as pigeons, grackles, and starlings. Discovery of the avian source has shed much light on situations involving sudden localized outbreaks of histoplasmosis. Infection typically results following the inhalation of minute airborne spores. Millions of people in the United States presently have or at one time were infected with either benign or acute respiratory histoplasmosis. The pulmonary disease closely mimics numerous other respiratory infections by eliciting an extremely wide range of signs and symptoms.

10-24. An unfortunate rare case progresses to severe disseminated or secondary histoplasmosis. As previously stated, *H. capsulatum* displays a marked affinity for the cells of the reticuloendothelial system. Fungal elements, upon reaching the bloodstream or lymphatics, are rapidly phagocytized by monocytes or macrophage cells. The organisms propagate and subsequently reach organs rich in reticuloendothelial tissue such as the liver, spleen, lymph nodes, and bone marrow.

10-25. In contrast to the direct examination of clinical materials in unstained wet mounts used when North American blastomycosis or paracoccidioidomycosis are suspected, *H. capsulatum* is best detected using Wright or Giemsa-stained smears of fresh material. Specimens such as smears from lymph node biopsy, cutaneous or mucocutaneous lesions, and bone marrow aspiration are best suited to this technique. Sputum, gastric washings, and other body fluids should be centrifuged and the sediment smeared and stained. These specimens should be collected in sterile containers; if they cannot be processed immediately (or require shipment), the addition of an antibiotic such as chloramphenicol not in excess of 0.2 mg./ml. is recommended. Any clinical material received by the laboratory for *H. capsulatum* culturing must be refrigerated if processing is delayed in order to retain the viability of the organism.

10-26. The stained organism in tissue form is a small, round, yeastlike cell, indistinguishable from many other similar structures which may be found in direct examination of clinical material. These yeastlike cells are readily phagocytised by mononuclear and occasionally polymorphonuclear cells, and thus when the typical intracellular form is seen it is of considerable diagnostic help. The organism within the phagocytic cell ranges from 1 to 4 microns in diameter. It is surrounded by a light blue ring representing its cell wall. The underlying cytoplasm stains a darker blue and contains chromatin material which stains a more intense ciolet color. Generally, this chromatin substance appears half-moon to crescent in shape.

10-27. Whether or not a morphological picture indicative of *H. capsulatum* is observed on the Wright or Giemsa-stained slide preparations, it is

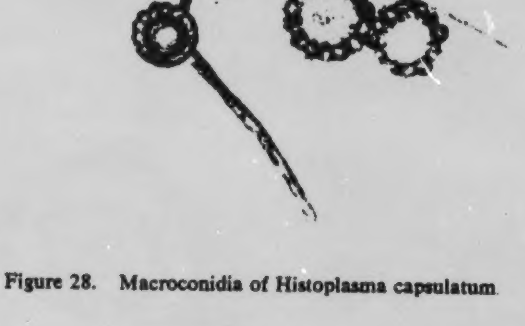


Figure 28. Macroconidia of Histoplasma capsulatum.

essential to attempt cultural recovery of the organism. The clinical material should be inoculated to several tubes of each of the various media used, to increase the likelihood of recovery. Tubes of brain-heart-infusion agar with antibiotics and Sabouraud's dextrose agar should be incubated at room temperature. Other tubes containing brain-heart-infusion blood agar (6 percent blood added to warm media prior to pouring) are incubated at 37° C. When attempting cultural recovery of *H. capsulatum* using screw-capped tubes, remember to loosen the cap ocasionally as this fungus is a strict aerobe. Another point worthy of re-emphasis is the fact that the yeast or tissue phase will not grow in the presence of antibiotics at 37° C. It is not advisable to use blood in media incubated at room temperature, because blood serves to inhibit the characteristic sporulation of the mycelial phase.

10-28. A distinct colony on Sabouraud's dextrose or brain-heart-infusion agar may not become discernable for at least 10 to 14 days at room temperature. *H. capsulatum* forms a white, fluffy colony with the typically fine and silky aerial mycelium seen in foldout 2, detail F–1. Gradually, after several weeks the entire slant surface becomes overgrown. It usually develops a buff to dark-tan color which usually coincides with sporulation. Microscopic examination of teased wet mount reveals a septate mycelium with both microconidial and macroconidial sporulation. The microconidia, or small spores, usually develop first and are borne sessile or on short stalks on the hypha. They are round to pyriform in shape, varying between 2 and 6 microns in diameter. Although usually smooth, a few echinulated spores may be seen. Some microconidia may be bicellular, thus being able to produce secondary spores by budding. The characteristic macronconidia which develop are large (7 to 25 microns in diameter), round to pyriform in shape, have a thick wall, and usually have a tuberculated surface. These tubercules are finger-like or spiny projections, which vary considerably in length on the same spore, ranging between 1 and 8 microns in length. Representative morphological features are shown in figure 28. Some strains of *H. capsulatum* do not produce tuberculate macronconidia, while the saprophytic species of the Genus *Sepedonium* produce macronconidia which are identical to those produced by some strains of *H. capsulatum*. Differentiation can be based on *H. capsulatum's* ability to convert to the yeast form at 37° C.

10-9. In order to confirm the cultural diagnosis, it is necessary to convert the mycelial growth phase of the fungus to the tissue form. This can normally be satisfactorily accomplished by inoculating some of the mycelial growth to tubes of brain-heart-infusion agar with a moist surface and incubating them at 37° C. If the agar surface appears dry, it may be moistened with sterile nutrient broth before use. Several subcultures may be necessary to effect a complete conversion. Most strains of *H. capsulatum* are convertible using this method. After 2 or 3 days' incubation, small, white to cream colored, round, convex colonies normally appear along the margin of the mycelial inoculum. These enlarge and eventually coalesce to form a moist, raised, soft growth (shown in FO 2, detail F–2), the surface of which varies from rough to granular. On occasion it may be highly mucoid. Microscopically, the yeast-phase cells are oval bodies (1.5 by 3.5 microns) which frequently display single and multiple budding and are indistinguishable from the fungus cells found in clinical material containing the tissue form of this pathogen.

10-30. Animal inoculation may be required to accomplish conversion of those strains of *H. capsulatum* which will not convert using the cultural technique. In such cases a 0.5-ml. suspension of spores or ground mycelium is inoculated intraperitoneally into white mice. Preparation of inoculum involves grinding a 4- to 6-week-old mycelial culture in sterile saline in a tissue grinder and adjusting the suspension to a density of a No. IV McFarland nephelometer. The mice are autopsied at intervals of 16 to 42 days after injection, and impression smears of both liver and spleen are examined for yeastlike cells. Regardless of the findings at autopsy, these tissues should then be cultured on appropriate media at 37° C. in a further attempt to obtain yeast-phase growth. Some mice which survive for several months may demonstrate a greatly enlarged spleen. The liver and spleen may be heavily infected with yeast cells.

10-31. Immunological tests have proven to be valuable tools in the diagnosis and prognosis of individuals with histoplasmosis. The histoplasmin skin test, as with other skin tests used for the diagnosis of systemic fungal diseases, is very useful in epidemiological studies. Its value in diagnosis is limited, however, because a positive response usually can indicate either a past or present infection. Skin sensitivity usually develops 4 to 8 weeks following infection but may decrease during the acute phase and disappear in terminal stages of the illness.

10-32. The complement-fixation and precipitin test are two serological techniques normally used for diagnosis and for following the course of histoplasmosis. The use of both histoplasmin and yeast-phase antigen is recommended when complement-fixation testing is performed. Titers should be determined as soon as the disease is suspected and again after at least a 6-week period. If a high titer results on the first sample and the followup titer is approximately as high or higher, the test is usually considered diagnostic. The precipitin test usually uses the antigen, histoplasmin, and is especially useful in early diagnosis of the acute pulmonary form of the disease. There is a need for careful standardization of test reagents, and best results usually require testing by experienced workers. Because of the variables encountered in serological tests, cultural diagnosis remains the best means of identifying disease due to *H. capsulatum*.

471

The Saprophytic Fungi

LOGICALLY, THE READER might ask "Since this volume is concerned with medical mycology, why must I be able to identify the saprophytic fungi which are present in cultures normally as a result of airborne contamination, or as contaminants of clinical material?" The answer is quite simple and just as logical. It is most important that the medical mycologist be able to distinguish saprophytic fungi from the pathogenic forms to prevent their being erroneously considered as the etiological agents of mycotic infection. It is not unusual for a particular saprophytic species to be isolated repeatedly from the same patient, and thus the inexperienced worker might presume it to be the causative disease agent.

2. The first section of this chapter will consider the most common laboratory contaminants with regard to their colonial morphology and microscopic structure. The majority of the saphrophytes which will be discussed are referred to solely by genus name since their various species are structurally so similar that speciation is possible only by experts in this particular area of mycology. The second section of this chapter will take up certain of the saprophytes that are encountered less commonly, although they can be important from the clinical standpoint.

11. Saprophytes Commonly Encountered in Medical Mycology

11-1. The saprophytes will be discussed in groups, each group having a particular microscopic structure useful in identification. Also, in each case the gross (macroscopic morphology) on Sabouraud's dextrose agar will be described. The various microscopic structures used to categorize these organisms include characteristic conidia, conidiophore, sporangiophores and, finally, the thallus (body).

11-2. **Saprophytes with Characteristic Conidia.** The common contaminants in order of presentation will include species belonging to the Genera *Alternaria, Cephalosporium, Curvularia, Fusarium, Helminthosporium, Nigrospora, Scopulariopsis,* and *Sepedonium.* The macroscopic morphological characteristics of these fungi are elaborated in table 25 and illustrated in foldout 2, detail G. Their microscopic morphological features are presented in table 26 and depicted in figures 29 through 36 by drawings made from slide cultures on potato dextrose agar.

11-3. *Alternaria spp.* is a rapid growing saprophyte which develops its mycelium close to the agar surface. It is grayish at first, later becoming black with a gray periphery; the underside or reverse pigment is black. The early aerial mycelium is sparce, but gradually areas of white, loose, cottony aerial mycelium develop. They become dull white to grayish in color and eventually may cover the black sporulating mycelium.

11-4. The macroconidia are typically uniform, having both transverse and longitudinal septations. The cells occur in chains from the ends of conidiophores and are dark brown in color. Each conidium is formed by the one immediately preceding it in the chain and demonstrates a dark spot at its point of attachment. Any cell comprising the conidium has the capability of elaborating a germ tube, and, thus, perpetuating the species. Frequently branching chains occur, since spore production is accomplished by successive budding of the most distal conidium in the chain.

11-5. *Cephalosporium spp.* is rapid-growing. At first it is deep rose in color, later becoming overgrown with a loose, white aerial mycelium. The flat colony which develops is glabrous to downy and wrinkled, and it displays a surface pigment which may vary from white to gray or pink. A reverse pigment is absent.

11-6. Microscopic examination reveals slender, unbranched conidiophores, which bear a spherical cluster of conidia at their tips. These conidia are usually single-celled but occasionally septations will result in conidia which display two or more cells. Production of conidia takes place at the apex of the conidiophore; clustering results as the conidia adhere to each other.

TABLE 25

MACROSCOPIC MORPHOLOGY OF COMMON SAPROPHYTIC FUNGI HAVING CHARACTERISTIC CONIDIA

Organism		*Growth Rate*	*Surface Topography*	*Surface Texture*	*Surface Pigment*	*Significant Back or Reverse Pigment*
Alternaria sp.		Rapid	Flat	Velvety to downy	Green to brown to irridescent black	Black
Cephalosporium sp.		Rapid	Flat	Glabrous to downy, wrinkled	White, gray, or pink	Absent
Curvularia sp.		Rapid	Flat	Cottony	Dark brown to black	Absent
Fusarium sp.		Rapid	Flat	Velvety to tight cotton	White, pink, or pale blue	Absent
Helminthosporium sp.		Rapid	Flat	Cottony	Young: Gray; Mature: Tan, gray-brown or gray-black	Absent
Nigrospora sp.		Rapid	Flat	Cottony to woolly	White to gray	Black
Scopulariopsis sp.		Rapid	Flat	Powdery to granular	White, tan, or brown	Absent
Sepedonium sp.		Rapid	Flat	Cottony	White	Absent

TABLE 26

MICROSCOPIC MORPHOLOGY OF COMMON SAPHROPHYTIC FUNGI HAVING CHARACTERISTIC CONIDIA

Organism	*Hypha*	*Conidiophore*	*Spore*
Alternaria sp.	Septate, dematiaceous	Simple, unbranched	Conidium: septate in two directions (muriform), dematiaceous, occur in chains with broad base of first conidium attached directly to conidiophore.
Cephalosporium sp.	Septate	Simple, branched or unbranched	Conidium: usually single celled (some show 2 - 3 cells) arranged in a clump or head of spores at end of each conidiophore.
Curvularia sp.	Septate, dematiaceous	Simple, unbranched	Conidium: large, thick-walled, multi-septate dematiaceous, curved more on one side than the other side.
Fusarium sp.	Septate	Simple, branched or unbranched, short or long	Conidium: multi-septate, banana or sickle shapted, in banana-like clusters on end of conidiophore; microconidium; oval or spherical, unicellular, or short, simple conidiophore.
Helminthosporium sp.	Septate, dematiaceous	Simple, branched or, unbranched, short, long, usually knotted	Conidium: multi-septate, thick-walled elliptical, and dematiaceous.
Nigrospora sp.	Septate	Simple, unbranched, short with inflated end or vesicle	Conidium: black spherical, borne on end of vesicle.
Scopulariopsis sp.	Septate	Simple, branched or unbranched	Conidium: lemon-shaped, echinulated, truncate base, occur in chains with truncate base of first conidium attached directly to conidiophore
Sepedonium sp.	Septate	Simple, unbranched, short or long	Conidium: spherical, thick-walled, with projecting spines or tubercles.

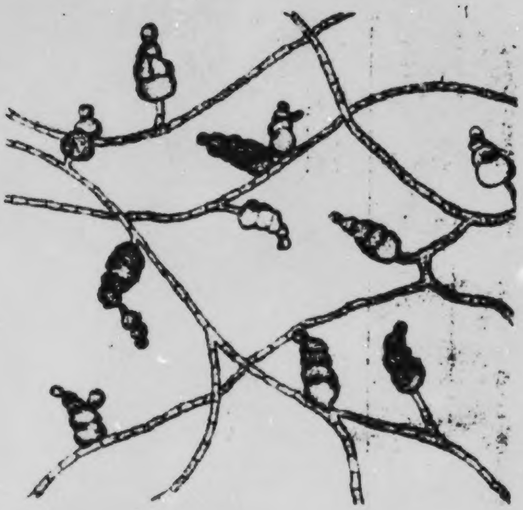


Figure 29. Alternaria.

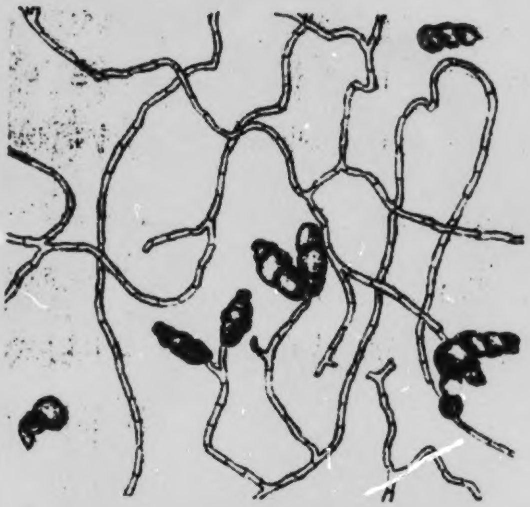


Figure 31. Curvularia.

11-7. *Curvularia spp.* produces a rapid-growing, flat, cottony-type colony which is dark brown to black in color. Reverse pigment is absent. Microscopically this fungus displays septate, dematiaceous (darkly pigmented) hyphae and simple, unbranched conidiophores. The conidia are large, thick-walled, multicellular, and also dematiaceous. Typically, one side of the conidium shows greater curvature than the other; thus, the genus name Curvularia seems most appropriate.

11-8. *Fusarium spp.* is a rapid-growing saprophyte which at first is white and cottony, but soon develops a deep rose color in the center. The pigment shades to a light pink at the periphery. When examined microscopically, this organism displays short, sepate hyphal branches giving rise to verticillate conidiophores. These abstrict (form by laying down a septum) long, fusoid or sickle-shaped, multiseptate macroconidia with pointed ends. Oval to spheroid unicellular microconidia are also produced on short, simple conidiophores.

11-9. *Helminthosporium spp.* rapidly develops a grayish colony which is flat and cottony. As it matures, the colony forms a matted, black, depressed central mycelium with a raised grayish periphery. Microscopically, the hyphae are septate and dematiaceous. The conidiophore may be simple, branched or unbranched, long or short, and is usually knotted. Numerous elliptical, multiseptate, thick-walled macroconidia develop from the conid-

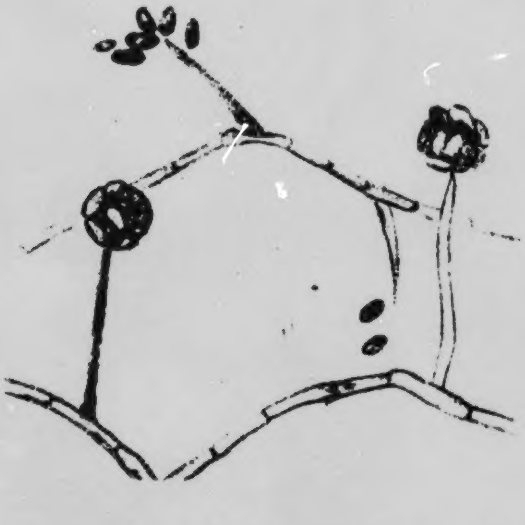


Figure 30. Cephalosporium.

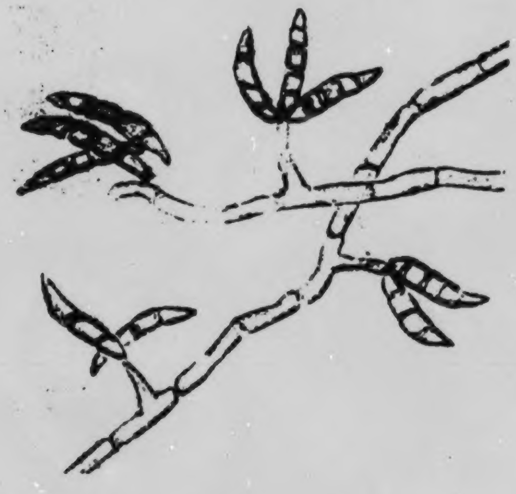


Figure 32. Fusarium.

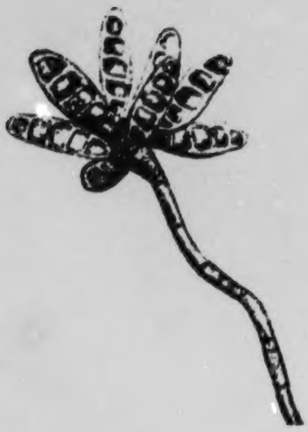


Figure 33. Helmenthosporium.

iophore. These macroconidia, like the hyphae, are dematiaceous (dark brown). When the macroconidia detach from the phore a dark scar is left at the former point of attachment. The wormlike shape of the conidiophore is no doubt responsible for the genus name *Helminthosporium*.

11-10. *Nigrospora spp.* is a rapid growing fungus that first develops a colony with compact, woolly, white aerial growth which gradually becomes gray due to the black, sporulating mycelium on the surface of the agar. The reverse pigment is black. Microscopic examination reveals septate hyphae from which develop simple, unbranched short conidiophores with swollen vesiclelike ends. The macroconidium which forms on the vesicle is black and spherical. When present in large numbers, it is responsible for the dark grayish colony color as well as the black reverse pigmentation.

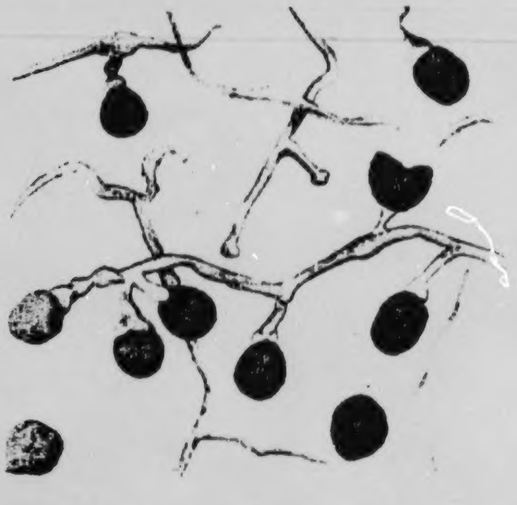


Figure 34. Nigrospora.

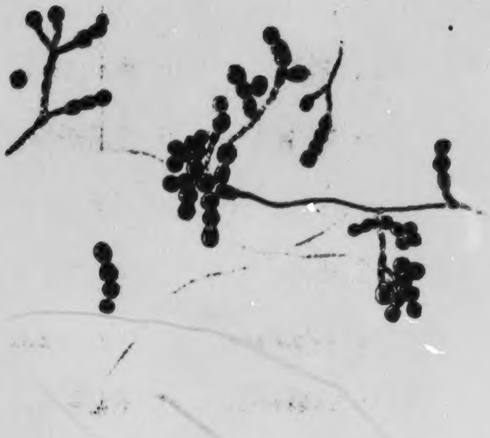


Figure 35. Scopulariopsis.

11-11. *Scopulariopsis spp.* is a slow-growing saprophyte at first membranous, wrinkled, and glabrous with regard to surface texture. As the colony matures aerial hyphae and conidia develop, giving the culture a powdery, light brown appearance. Microscopically, the hyphae are septate and the conidiophores are simple—either branched or unbranched. The lemon-shaped, echinulated conidia which develop in unbranching chains on the condiophores have a characteristic pointed apex and truncate (cut off) base.

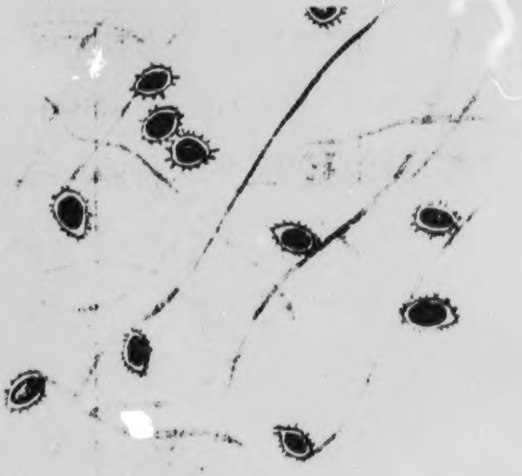


Figure 36. Sepedonium.

TABLE 27

MACROSCOPIC MORPHOLOGY OF COMMON SAPHROPHYTIC FUNGI HAVING CHARACTERISTIC CONDIOPHORES

Organism	*Growth Rate*	*Surface Topography*	*Surface Texture*	*Surface Pigment*	*Significant Back or Reverse Pigment*
Aspergillus clavatus	Rapid	Flat	Woolly	Blue to green	Absent
Aspergillus fumigatus	Rapid	Flat	Velvety or powdery	Gray-blue to green, dark	Absent
Aspergillus glaucus	Rapid	Young: flat mature: slightly heaped	Young: floccose mature: granular	Young: white mature: green center with yellow-gold periphery	Absent
Aspergillus niger	Rapid	Flat	Woolly	Young: white to yellow mature: dark brown to black	Absent
Cladosporium sp.	Rapid	Flat to slightly heaped	Glabrous, downy, or velvety	Green to black, dark	Green to black, dark
Paecilomyces sp.	Rapid	Flat	Powdery to velvety	Yellow-brown, gray-green, violet, or white	Absent
Penicillium sp.	Rapid	Flat	Powdery	White-green to blue and other colors	Absent
Trichoderma sp.	Rapid	Flat	Woolly	White to green	Port wine to dark brown soluble pigment

TABLE 28

MICROSCOPIC MORPHOLOGY OF COMMON SAPHROPHYTIC FUNGI HAVING CHARACTERISTIC CONIDIOPHORES

Organism	*Hypha*	*Conidiophore*	*Spore*
Aspergillus clavatus	Septate, branched.	Consists of stalk, vesicle and sterigmata; stalk expands into *clavate* vesicle; single rows of sterigmata over *entire* surface of vesicle; echinulated stalk (oil).	Conidium: smooth, gray-green occur in unbranched chains from sterigmata.
Aspergillus fumigatus	Septate, branched.	Consists of stalk, vesicle and sterigmata; stalk expands into *flask-shaped* vesicle; single rows of sterigmata occur on *upper half* of vesicle; smooth stalk.	Conidium: thick echinulated, dark green; occur in unbranched chains from sterigmata.
Aspergillus glaucus	Septate, branched.	Consists of stalk, vesicle and sterigmata; stalk expands into *globous* vesicle; single rows of sterigmata occur over *entire* surface of vesicle; smooth stalk.	Conidium: (asexual); echinulated, green; occur in unbranched chain from sterigmata; ascospore (sexual), average of 8 within asci, asci within perithecium.
Aspergillus niger	Septate, branched.	Consists of stalk, vesicle and sterigmata; stalk expands into vesicle having usually *two* (1-2) rows of sterigmata over *entire* surface of vesicle; smooth stalk.	Conidium: echinulated, dematiaceous; occur in unbranched chains from sterigmata.
Cladosporium sp.	Septate, branched.	Simple, branched, produces long branched chains of microconidium (cladosporium-type sporulation).	Conidium: occur in branched chains.
Paecilomyces sp.	Septate, branched.	Consists of single or multiple (often in whorles) sterigmata from which chains of microconidia originate.	Conidium: elliptical, occur in unbranched chains which appear to slightly bend in one direction along with conidiospore.
Penicillium spp.	Septate, branched.	Simple: includes stalk and sterigmata; complex: includes stalk, metulae, and sterigmata; sterigmata are brush like; variations between simple and complex conidiophores are species specific.	Conidium: smooth or echinulated, elliptical; occur in unbranched chains from sterigmata.
Trichoderma sp.	Septate, branched.	Simple, branched; sterigmata are alternating or directly opposed on short hyphal stalk of conidiophore.	Conidium: spherical, formed in clusters at tip of sterigmata.

11-12. *Sepedonium spp.* grows rapidly, forming a flat, cottony colony which displays a white surface pigment. There is no reverse pigment. Microscopic study reveals septate hyphae, which elaborate simple long or short unbranched conidiophores. The macroconidia are spheical and thick-walled with projecting spines or tubercles. This saprophyte, as noted in an earlier chapter, closely simulates some strains of *Histoplasma capsulatum.*

11-13. **Saprophytes with Characteristic Conidiophores.** The saprophytic fungi which comprise the common laboratory contaminants of this group include members of the Genera *Aspergillus, Cladosporium, Paecilomyces, Penicillium,* and *Trichoderma.* The macroscopic morphological characteristics of these fungi are given in table 27 and illustrated in foldout 3, detail A. Their microscopic morphological features are presented in table 28 and shown in figures 37 through 41 in drawings from slide cultures.

11-14. *Aspergillus clavatus,* a rapid-growing saprophyte, develops a flat colony with a woolly surface texture. It has a blue-green surface pigment and lacks any reverse pigmentation. The hyphae are branched and septate. The conidiophore is composed of a stalk, vesicle, and sterigmata. The stalk arises from a foot cell and expands at its apex to form a clavate vesicle covered with single rows of sterigmata. Echinulations may be observed on the stalk under oil immersion. The microconidia are smooth, gray-green in color and occur in unbranched chains from the sterigmata.

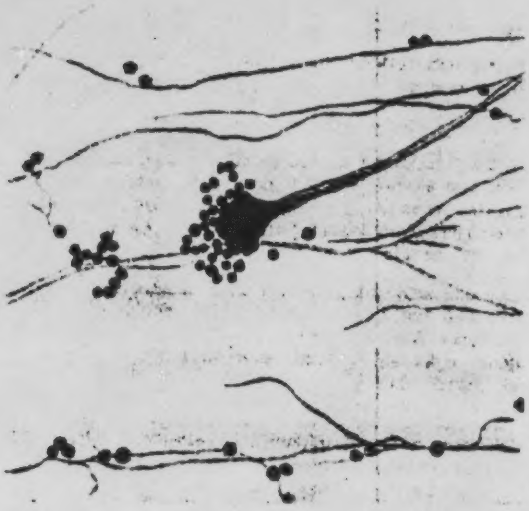


Figure 37. Aspergillus.

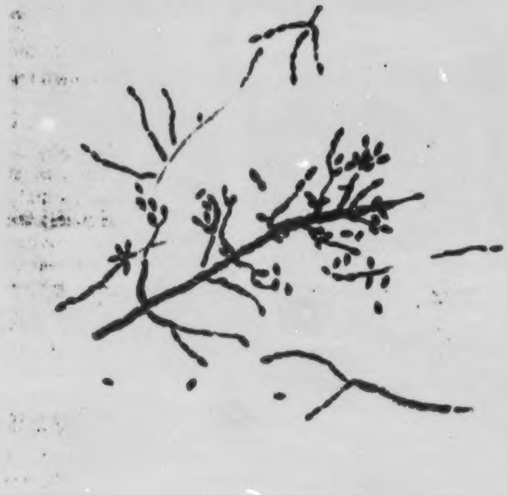


Figure 39. Paecilomyces.

11-15. ***Aspergillus fumigatus***, a rapid-growing contaminant, forms a flat colony with a velvety or powdery texture. The surface pigment is a dark gray-blue to green and reverse pigmentation is absent. Microscopically, *A. fumigatus* has a flask-shaped vesicle which sets it apart from *A. clavatus*. Single rows of sterigmata occur only on the upper half of the vesicle and the stalk is smooth. Microconidia are thick, echinulated, and dark green in color. They occur in unbranched chains from the sterigmata.

11-16. The ***Aspergillus glaucus*** species of *Aspergillus* is rapid-growing, and at first forms a flat colony which later becomes slightly heaped. The immature colony is woolly in texture, later becoming granular. The young colony is white, while the mature growth shows a green center with a yellow-gold periphery. There is no reverse pigment. *A. glaucus* microscopically reveals a glabrous vesicle covered with single rows of sterigmata. The stalk is smooth. The green and echinulated microconidia occur in unbranched chains from the tips of the sterigmata.

11-17. ***Aspergillus niger*** is a rapid-growing species that forms a flat woolly colony which is at first white to yellow. As the colony matures, it becomes dark brown to black. The vesicle of *A. niger* usually has two rows of sterigmata over the entire surface and the stalk is smooth. The microconidia are echinulated and dematiaceous, occur-

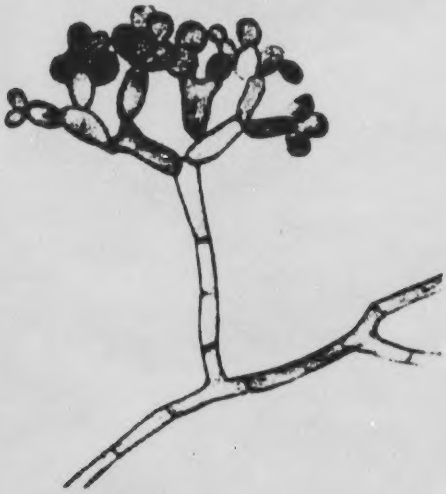


Figure 38. Cladosporium.

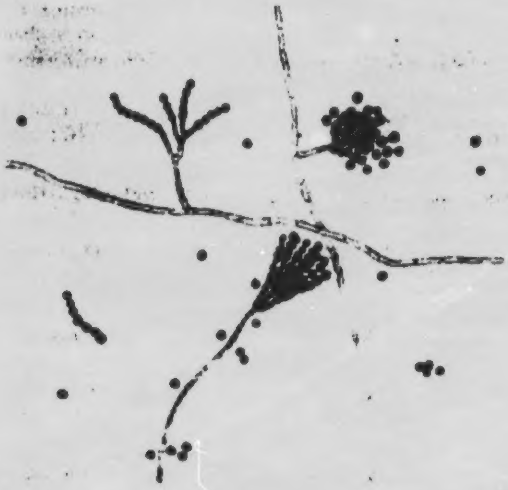


Figure 40. Penicillium.

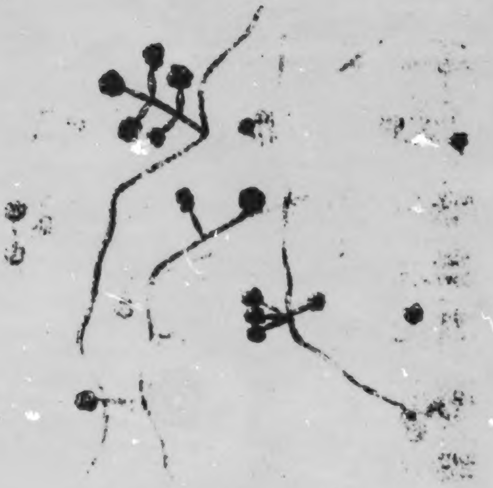


Figure 41. Trichoderma.

ring in unbranched chains from the tips of the sterigmata.

11-18. *Cladosporium spp.* is another rapid-growing saprophyte that develops a flat to slightly heaped colony which may be glabrous, downy, or velvety. Both the front and reverse pigmentation range from dark green to black. The hyphae are septate and branched under the microscope. The conidiophore is simple and branched, forming long branching chains of continuously budding microconidia. The microconidia may septate and becom bicellular when mature. The hyphae, conidiophores, and conidia are dark brown in color.

11-19. *Paecilomyces spp.* is a rapid-growing contaminant producing a flat colony with a powdery or velvety surface texture. The surface pigmentation may be yellow-brown, gray green, violet, or white, and reverse pigment is absent. The microscopic picture is one of septate branched hyphae with conidiophores existing either singly or in groups (often in whorls). Flask-shaped sterigmata with elongate, condial-bearing tubes may be seen on the conidiophores. The elliptical micronconidia occur in unbranched chains and, along with the conidiophores and sterigmata, bend away from the main axis of the hypha.

11-20. *Penicillium spp.* is a rapid-growing familiar saprophyte that develops a flat powdery colony which is generally white-green blue in color. There is no reverse pigmentation. Microscopically, the genus *Penicillium* typically reveals hyphae which are septate and branched. Conidiophores may be either simple or complex and are indicative of particular species. The simple conidiophore consists of only a single stalk and sterigma from which microconidia are elaborated in an unbranched chain. The complex condiophore includes a stalk which branches out apically in the form of a brush. Each branch develops a metula, within which conidia are generated; and a flask-shaped sterigmata, through which the spores are passed in unbranching chain arrangement. The elliptical microconidia may be either smooth or echinulated.

11-21. *Trichoderma spp.*, the last member of this group of saprophytes with characteristic conidiophores, also grows rapidly to form a flat, woolly colony which varies from white to green in color. Reverse pigmentation ranges from port wine to a dark brown. As with all the previous members of this group the hyphae are septate and branched. The simple branched conidiophores may alternate or directly oppose each other. The spherical microconidia are extended through a sterigmata forming a loosely adherent globular cluster at its tip.

11-22. **Saprophytes with Characteristic Sporangiophores.** Those common contaminants which belong to the Class *Phycomycetes* will be described from both a macroscopic and microscopic standpoint in this section. They include species of the Genera *Absidia, Mucor, Rhizophus,* and *Syncephalastrum*. The macroscopic features of these fungi are available in table 29. Refer to figures 42 through 45 and table 30 for data regarding the microscopic morphology of each member of this group.

11-23. *Absidia spp.* is a rapid-growing saprophyte that forms a flat colony which is woolly and coarse in texture and displays a surface pigment varying from white to gray, brown, or yellow. Reverse pigmentation is absent. Under the microscope the hyphae are broad and aseptate. Rhizoids develop at the nodes. The conidiophore is comprised of a stalk which swells apically to form the spore-generating organ called the columella and pear-shaped, saclike, sporangium which incloses the columella. The sporangiospores (endospores) when released from the columella are entrapped in the sporangium.

11-24. *Mucor spp.* is a rapid-growing contaminant that forms a flat colony which is also woolly and coarse. The surface pigmentation ranges from white to gray, brown, or yellow, and no reverse pigmentation is present. The hyphae are broad and aseptate. The conidiophore is essentially the same as with *Absidia spp.* except that the sporangium is spherical in shape. Frequently, there is branching of one conidiophore from another. The sporangiospores are identical to those produced by Absidia spp.

11-25. *Rhizopus nigricans*, another rapid-growing fungus, forms a flat colony which is woolly in texture. Surface pigmentation is gray to brown and

TABLE 29

MACROSOPIC MORPHOLOGY OF COMMON SAPROPHYTIC FUNGI (PHYCOMYCETES) HAVING CHARACETRISTICS SPORANGIOPHORES

Organism	*Growth Rate*	*Surface Topography*	*Surface Texture*	*Surface Pigment*	*Significant Back or Reverse Pigment*
Absidia sp.	Rapid	Flat	Woolly, Coarse.	White to gray, brown, or yellow	Absent
Mucor sp.	Rapid	Flat	Woolly, Coarse.	White to gray brown, or yellow	Absent
Rhizopus nigricans	Rapid	Flat	Woolly	Gray to brown, dotted with black sporangia	Absent
Rhizopus oryzae	Rapid	Flat	Woolly	White to gray or brown	Absent
Syncephalastrum sp.	Rapid	Flat	Woolly	White to dark gray	Absent

NOTE: The macroscopic morphology of these Phycomycetes resembles the macroscopic morphology of *Nigrospora* sp., seen in plate VII.

dotted with black sporangia. There is no reverse pigmentation. The microscopic picture reveals broad aseptate hyphae. The conidiophores develop in nodal groups at opposite ends of hyphal runners called stolons. These conidiophores form a spherical sporangium and are essentially the same as those previously described. A tuft of root like hyphae (rhizoid) develops at the ends of the stolon opposite the conidiophore and aids the fungus in adhering to the substratum as well as facilitating assimilation of nutrient material. Except for their black color, the sporangiospores appear the same as those of *Absidia* and *Mucor spp.*

11-26. The growth rate and macroscopic morphology of *Rhizopus oryzae* cannot be distinguished from that of *R. nigricans*. Microscopically, the only variation from *R. nigricans* involves the stalk of the conidiophore (which is striated) and the sporangiospores, which are dark brown rather than black.

11-27. *Syncephalastrum spp.* is a rapid-growing contaminant that is essentially the same macroscopically as *R. oryzae*. Microscopic examination reveals the characteristic broad, aseptate hyphae of the Class *Phycomycetes*. The complex conidiophores consist of short branches of aerial hyphae which are greatly swollen at their tip-forming vesicles. These vesicles bear many elongate fingerlike projections, the sporangia, which produce sporan-

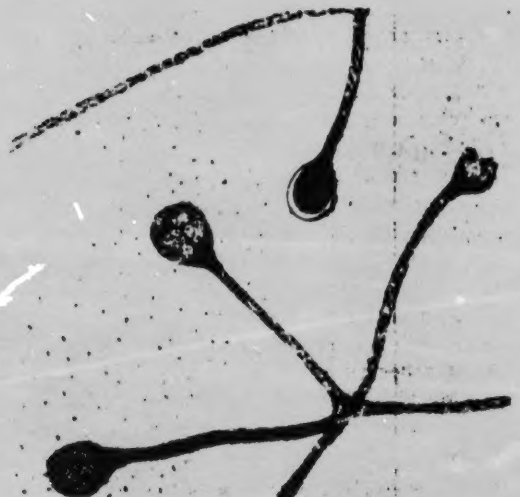


Figure 42. Absidia.

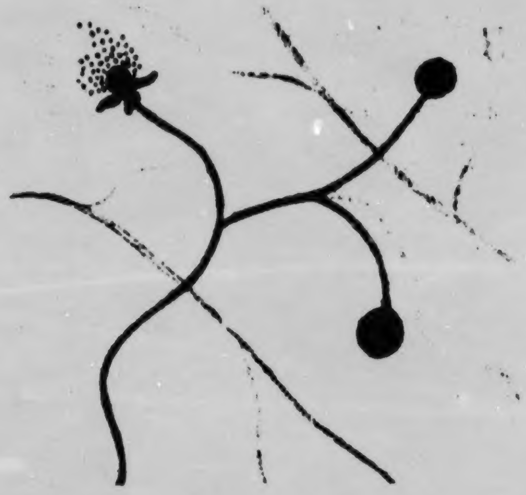


Figure 43. Mucor.

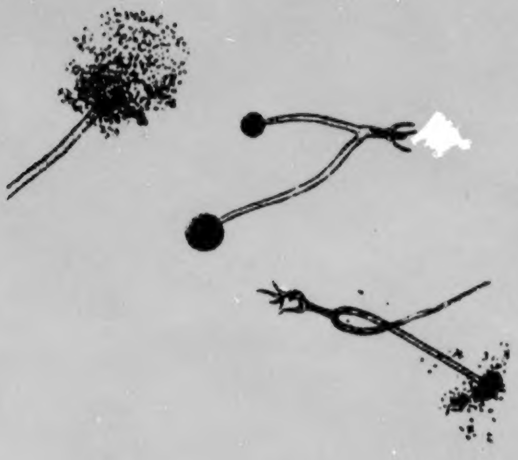


Figure 44. Rhizopus.

giospores in long chains. When observed under low magnification, this fungus may be readily mistaken for a species of *Aspergillus.*

11-28. **Saprophytes with Characteristic Thallus.** This final group of common contaminants reproduce by forming spores within or directly from the plant body. The members which will be discussed in this section include species belonging to the Genera *Aureobasidium, Geotrichum, Rhodotorula,* and *Streptomyces.* Refer to table 31 for information regarding their macroscopic morphological characteristics and foldout 3, detail B, for illustration of their colonial morphology on Sabouraud's dextrose agar. Table 32 is provided as a ready reference to their microscopic morphology.

11-29. *Aureobasidium pullulans* is a rapid-growing fungus that forms a heaped, wrinkled colony which varies in texture from pasty to glabrous or leathery. The surface pigment is typically black, with a grayish-pink fringe of submerged mycelial growth. The reverse pigment, when present, is black. In its early stages of development the hyphae appear thin, hyaline, and septate. As the hyphae mature, large, black (highly dematiaceous), thick-walled cells are formed. The microconidium may bud directly from the walls of young hyphae, or develop on short germ tubes elaborated by the older hyphal cells.

11-30. *Geotrichum spp.* is a rapid-growing saprophyte that forms a flat colony whose surface texture is at first pasty, later becoming velvety and cottony. The surface color varies from gray to tan and no reverse pigment is developed. The microscopic picture is one of long, slender, multiseptate hyphae which readily fragment into thin-walled, rectangular arthrospores. These arthrospores are reproductive bodies which proliferate germ tubes to form new hyphal filaments.

11-31. *Rhodotorula spp.* produces a colony which develops rapidly. The colony is flat and displays a pasty to mucoid surface texture. The surface pigment is orange to red, and when produced, the reverse pigmentation is an orange-red. There are no hyphae or conidiophores developed by this fungus. Reproduction is by means of simple budding of yeast type cells called blastospores.

11-32. In contrast to the great majority of saprophytic fungi, *Streptomyces spp.* is slow in developing. The colony is heaped and somewhat wrinkled—presenting a dry, chalky, leathery appearance on the medium. Surface pigmentation varies considerably, depending on the species. It ranges from cream to tan or brown. There is no reverse pigmentation. The long, slender, branching hyphae (less than 1 micron in diameter) fragment into chains of microconidia. These chains are very delicate and morphological studies usually are best accomplished by means of slide cultures.

12. Saprophytes as the Etiological Agents of Rare Mycoses

12-1. Some of the previously discussed saprophytic fungi have been implicated in serious mycotic infections in the tissues of man and animals. Whether these organisms are the actual causative agents of disease, or are present simply as contaminants, is often difficult to assess. It is well to remember that these saprophytes may be present in the role of secondary invaders, particularly in cases of tuberculosis. Typically these organisms are opportunistic, becoming pathogenic only under favorable conditions which may occur in people

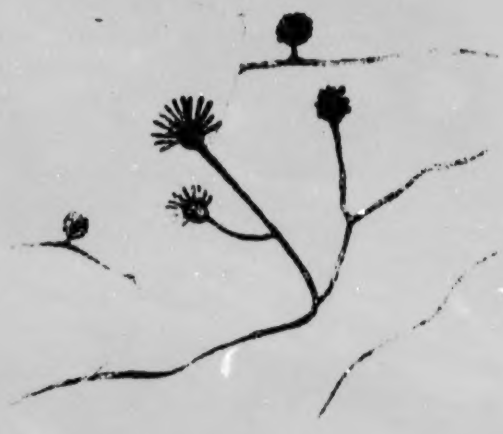


Figure 45. Syncephalastrum.

TABLE 30

MICROSCOPIC MORPHOLOGY OF COMMON SAPROPHYTIC FUNGI (PHYCOMYCETES) HAVING CHARACTERISTIC SPORANGIOPHORES

Organism	*Hypha*	*Sporangiophore*	*Spore*
Absidia sp.	Aseptate, rhizoids at nodes.	Consists of stalk, columella and pear-shaped sporangium, arise in groups, internodally.	Sporangiospore, borne in group upon columella and surrounded by sporangium.
Mucor sp.	Aseptate, branched.	Consists of stalk, columella and spherical sporangium; usually branched.	Same as above.
Rhizopus nigricans	Aseptate, rhizoids at nodes.	Consists of stalk, columella and spherical sporangium; arise in groups from node.	Sporangiospores are black, otherwise, same as above.
Rhizopus oryzae	Aseptate, rhizoids at node.	Stalk is striated, otherwise same as rhizopus nigricans.	Sporangiospores are dark brown; otherwise, same as above.
Syncephalastrum sp.	Aseptate, branched.	Complex, producing many clavate sporangia from vesicle, often short and curved.	Sporangiospores formed in clavate sporangium.

with chronic debilitating diseases or whose normal immune response has been impaired.

12-2. In theory, any of the saprophytes is capable of causing disease in man. The *Genus Aspergillus, Penicillium* and several members of the class *Phycomycetes* have been implicated in the majority of the cases. The laboratory diagnosis of mycoses resulting from these normally innocuous fungi along with those agents causing cladosporiosis and rhinosporidiasis, will be discussed in this section.

12-3. **Aspergillosis.** This infrequently occurring mycosis caused by various species of *Aspergillus,* is typically a granulomatous, necrotizing, cavitary disease of the lungs. On occasion, inflammatory lesions occur in the skin, external ear, nasal sinuses, and the eye. In disseminated form the bones, meninges, and other body organs may become involved. Species of Aspergillus are ubiquitous in nature, and infections have been reported from all areas of the world. Plants, insects, domestic animals, and especially birds are susceptible to aspergillosis. Agricultural workers who are exposed to massive doses of spores, frequently contract the primary pulmonary form of the disease often referred to as "farmers lung." A particular species of *Aspergillus* generally is associated with a specific infection site. Usually, strains of *A. fumigatus* are isolated from cases of pulmonary aspergillosis, while *A. niger* is the most common species causing external ear infections. The various clinical forms include pulmonary, cutaneous, disseminated, and on rare occasion rhino-ocular infections.

12-4. Primary pulmonary aspergillosis, the most common type of human infection, elicits symptoms which closely simulate those of pulmonary tuberculosis. Individuals who live or work in environments where *A. fumigatus* spores are prevalent may become sensitizied; and, thus, when a large number of spores are inhaled they develop an allergic asthma. Species other than fumigatus may also produce sensitization. The most common form of cutaneous aspergillosis is otomycosis, an external ear infection, with *A. niger* as the usual etiological agent. Skin lesions may also result from bloodstream dissemination in cases of fulminating systemic aspergillosis. A few cases of mycetoma (fungal tumor) due to *A. amstelodami* and *A. nidulans* and other species have been reported in the literature.

TABLE 31

MACROSOPIC MORPHOLOGY OF COMMON SAPROPHYTIC FUNGI HAVING CHARACTERISTIC THALLI

Organism	*Growth Rate*	*Surface Topography*	*Surface Texture*	*Surface Pigment*	*Significant Back or Reverse Pigment*
Aureobasidium pullulans (pullularia pullulans)	Rapid	Flat	Pasty to glabrous to leathery	White or pink, to black	Absent or black
Geotrichum sp.	Rapid	Flat	Pasty to velvety to cottony	Gray to tan	Absent
Rhodotorula sp.	Rapid	Flat	Pasty to mucoid	Orange to red	Absent or orange-red
Streptomyces sp.	Rapid	Heaped	Dry, chalky, leathery	Cream, tan or brown	Absent

TABLE 32
MICROSCOPIC MORPHOLOGY OF COMMON SAPROPHYTIC FUNGI HAVING CHARACTERISTIC THALLI

Organism	*Hypha*	*Sporophore*	*Spore*
Aureobasidium pullulans (Pullularia pullulans)	Septate, thin, hyaline (young) developing into wide, thick-walled dematiaceous (old) hypha.	Conidiophore: not prominant.	Microconidium: may bud from young hypha or grom germ tube of old hypha.
Geotrichum sp.	Multiseptate, fragmented into arthrospores.	None	Arthrospore: square to oval segments from fragmented, multiseptate hypha.
Rhodotorula sp.	None	None	Blastospore: budding yeast-type cell.
Streptomyces sp.	Slender, fine (1 micron or less, dia.), branching.	Straight or coiled, breaks up into chains of microconidia.	Microconidium: occurs in chains

12-5. Disseminated aspergillosis normally results from the extension of a primary pulmonary infection which fails to be contained by calcification in the lungs. The fungus is spread to the vital organs of the body via the bloodstream, resulting in widespread tissue necrosis and severe toxemia. *A. fumigatus*, *A. flavus*, and other species have on rare occasion invaded the nasal sinuses, the orbit of the eye and the eye itself, resulting in the rhino-ocular form of aspergillosis. This form of the disease often spreads to the central nervous system.

12-6. Since species of Aspergillus are so frequently observed in and cultured from clinical materials, their implication in disease processes normally requires repeated recovery from clinical materials in the absence of other pathogenic agents and positive histologic findings. Recovery of the organism from unexposed specimens, such as pleural or spinal fluid is strong supportive evidence.

12-7. Direct wet preparations should be accomplished on sputum, bronchial washings, and other body fluids. When volume is sufficient, it is best to use the centrifuged sediment. The mycelium appears wide, 4 to 6 microns in diameter, of a fairly uniform width, and septate. In the absence of a conidial head it is not possible to distinguish *Aspergillus* from morphologically similar fungi; however, if large quantities of this type of hyphae are present, it is quite suggestive.

12-8. The material must be cultured on media such as Sabouraud's dextrose agar containing antibacterial agents other than cycloheximide. Most *Aspergillus* isolates are sensitive to this antibiotic. As soon as growth is adequate, wet mounts are prepared. *A. fumigatus* has been identified as the etiological agent of aspergillosis in 90 to 95 percent of such cases. Animals have been used by several researchers to demonstrate the thermolabile endotoxin produced by *A. fumigatus*. Animal inoculation techniques are considered unnecessary, however, in identifying the Aspergilli. Present immunologic tests are of no value because of the frequency of cross reactions and the lack of standardized reagents.

12-9. **Phycomycosis (Mucormycosis).** Several genera of the class *Phycomycetes* are capable of causing this rapidly fatal disease characterized by acute inflammation and vascular thrombosis. Since the name mucormycosis refers only to species and strains belonging to this genus, the use of the term "phycomycosis" seems much more appropriate. Genera other than *Mucor* which may be implicated in phycomycoses include *Absidia, Rhizopus, Mortierella,* and *Basidiobolus.* These ubiquitous fungi, as in the case of Aspergilli, are common laboratory contaminants. Unless they are repeatedly isolated from clean clinical material and the findings corroborated culturally and by histologic examination, a definitive diagnosis is difficult. Phycomycosis has been reported from many areas of the world, and it is likely that the disease, although rare, is cosmopolitan in distribution. Systemic infections occur either by inhalation or ingestion of spores, or in localized cutaneous form as a result of skin penetration. There are several conditions predisposing to a phycomycosis, and probably the most important of these is diabetes mellitus. Such conditions as malnutrition, uremia, and amebiasis preceding visceral phycomycotic infections have been reported. Another increasingly significant predisposing factor is the prolonged use of antibiotics and steroids. The phycomycoses have been classified into four basic types: cranial-facial, thoracic, abdominal-pelvic, and dermal.

12-10. The cranial-facial form of infection, usually affecting patients with severe acidotic diabetics, often originates in the paranasal sinuses. Thoracic phycomycosis results from inhalation of spores or aspiration of fungal material draining from lesions either in the nose or mouth. It is characterized by progressive nonspecific bronchitis and pneumonia. Since the fungi demonstrate a particular affinity for the blood vessels, they are often found in large numbers in their lumens so that thrombi and resultant infractions are commonplace.

12-11. The abdominal-pelvic form of infection presumably originates from the ingestion of infective fungal elements, or possibly due to bloodstream dissemination. No particular region of the alimentary tract appears to be more susceptible than another. A few well-documented cases of subcutaneous phycomycosis have been reported from Indonesia. The histories in these cases did not indicate injury to the skin; however, they did indicate that the infection began at one spot as a subcutaneous nodule which gradually increased in size. Healing takes place without specific treatment after periods of several months to a few years. The organisms proved to be *Basidiobolus ranorum.*

12-12. For diagnosis of a phycomycosis clinical materials aspirated from nasal sinuses, or obtained by the scraping of nasal or oral lesions, should be examined directly in potassium hydroxide for the presence of broad (6 to 15 microns in diameter), aseptate, branching hyphae. A rare septation may be observed. When body fluids are submitted, such as spinal fluid or bronchial washings, they should be concentrated by centrifugation before examination. The clinical material should be cultured on Sabouraud's dextrose agar without cycloheximide and incubated at 25° C. If blood or pus is present in the specimen, give this part of the specimen priority for use in inoculation of the medium. The *phycomycetes* are fast-growing; they rapidly fill the tube with a white, cottony growth which turns gray with sporangial formation. Teased amounts in lactophenol cotton blue are examined microscopically for broad aseptate hyphae, sporangia, and spores. Genus identification should be based on structural features such as rhizoids, stolons, or conidial variations. The characteristics of the common agents of phycomycosis are available in tables 29 and 30.

12-13. The most rapid method of diagnosing a phycomycotic infection is to prepare a frozen section of biopsied tissue stained with hematoxylin and eosin. The fungus appears in the tissue as broad, branched, aseptate hyphae which may reach 200 microns in length. Culturing is necessary for genus and species identification. Animal inoculation and immunologic procedures are of no value.

12-14. **Rhinosporidiosis.** *Rhinosporidium seeberi* is the fungus responsible for this worldwide human disease. It is a chronic, granulomatous mycosis characterized by the development of polyp-like masses, primarily of the mucous membranes. In more than 90 percent of reported cases, the polyps first appeared on the mucous membranes of the nose, either sessile or pedunculated, and often of sufficient size to obstruct breathing. The eyes are frequently involved. Many such cases probably go undiagnosed because of early surgical removal without histological study. Occasional infections of the ears, larynx, and genitalia have also been reported. The natural habitat of the organism is unknown and, therefore, the source of infection is obscure. Cases of the disease in India and Ceylon have been associated with swimming and working in stagnant water, and the theory has been advanced that aquatic animals, such as fish or frogs, may host the fungus.

12-15. The polyps which develop are soft, red, and lobed. They often resemble the surface of a cauliflower. Small white spots seen on the surface of the polyp are actually mature sporangia produced by the organism. Microscopic examination of these polyps reveals spherical bodies varying in size between 6 and 300 microns. These bodies represent the progressive stages in the organism's development. As these sporangia enlarge, they develop thick refractile outer walls, and the cytoplasm undergoes progressive cleavage to form thousands of lobated endospores. When mature, the sporangial wall ruptures and releases the endospores into the surrounding tissue where they enlarge to form new sporangia.

12-16. *Rhinosporidium seeberi* has not been cultured and, thus, its exact taxonomic position has never been established. Its close resemblance to the sporangial development seen in the *Phycomycetes* suggests that it probably is a member of the class *Phycomycetes.* Other factors supporting this classification are the chitinous and cellulose components of the sporangial walls; these constituents are also found in the cell walls of some of the lower Phycomycetes.

12-17. Laboratory identification of rhinosporidiosis is dependent on clinical observation, and histologic examination of biopsied tissue. The sporangia can be seen in unstained preparations with the naked eye; they appear as large cavities in the surrounding tissue. Special staining techniques are not required; however, the periodic-acid-Schiff and Gridley stains are very effective.

12-18. There is a possibility that these sporangia may be mistaken for the spherules of *Coccidioides immitis* when examined microscopically. Differentiation of the two may be made on the basis of size, since the mature sporangia of *R. seeberi* attain considerably greater size than the mature spherules of *C. immitis.* Also the endospores of *R. seeberi* are lobulated and stain darkly, while those of *C. immitis* are nonlobulated, with only the endospore wall staining well.

12-19. **Cladosporiosis.** Of the three *Cladosporium* species which cause human disease only *Cladosporium bantianum* should be referred to as causing *Cladosporiosis. C. carrionii* is an agent of chromoblastomycosis, while *C. werneckii* was covered in the chapter concerned with the monomorphic molds. The organism, *C. bantianum,* has been referred to in the past as *Cladosporium trichoides* and *Torula bantiana.*

12-20. The fungus causes a brain abscess in man, but its portal of entry remains a mystery. Purulent material from the lesion reveals brown, aseptate branched hyphae 1 to 2 microns in diameter with many vesicular chlamydospores that reach a diameter of 8 microns. Macroscopically, on Sabouraud's dextrose agar, growth is slow. Flat to some dome-shaped colonies reach a diameter of about 3 cm. develop after 2 weeks. The surface is velvety and may form radial folds. Front pigmentation is dark olive gray to black, while the growth is jet black when viewed from the back.

12-21. Teased mounts of the growth microscopically show a brown septate mycelium from which develop simple or septate conidiophores bearing long, sparsely branched chains of conidia. The conidia are elongate and brown, having dark disjunctors between them or on each end of the free spores. The conidia are budded from the most distal cells, causing these to be the smallest in the chain.

12-22. Laboratory identification of *C. bantianum* is based on morphologic and physiologic criteria. The morphologic features of this fungus are elaborated in the preceding paragraphs. *C. bantianum* is morphologically similar to *C. carionii*, but can be distinguished by its more rapid growth rate, the extreme length of its spore chains, the development of longer conidia and its ability to grow at temperatures as high as 42 to 43° C. *C. bantianum* and *C. carionii* can be distinguished from the saprophytic *Cladosporium* species on the basis of their slower growth rate and their inability to liquefy gelatin.

CHAPTER 5

Virology

IT WASN'T LONG ago that the clinical laboratory worker's familiarity with virus technology did not need to extend beyond the occasional collection of blood serum to be sent off to a reference laboratory for antibody studies. Today we not only have a wider variety of sensitive serological test systems, but the development of tissue culture methods for growing viruses has greatly simplified the problem of isolating these disease agents in the laboratory. As a consequence, the trend in medical practice is toward greater use of diagnostic procedures offered by the virologist to confirm clinical impressions regarding the viral nature of an infection.

2. You will still find, however, that few military hospitals have the facilities and specially trained personnel required to carry out lengthy virological procedures—especially virus isolations. Hence, we continue to rely upon consultant centers or epidemiological laboratories that are appropriately equipped and staffed to handle viruses. The noticeable change in hospital laboratory work patterns comes from the greater diversity and volume of specimens submitted for examination—and the care that must be taken to preserve the viability of viruses in clinical materials so that isolation attempts will be successful.

3. Accordingly, the main purpose of this chapter is to set forth general guidelines for the collection, preservation, packaging, and shipment of clinical specimens. The other material included in the chapter is designed to build an understanding of why these guidelines are important.

4. Following a brief exploration of the significance of viruses and related pathogens, we will report recent findings on their nature and mode of reproduction. Then we will take a look at characteristics of the major groups and the diseases they cause. After that, we will discuss collection and shipment techniques. Lastly, there will be a short resume of the conventional procedures used by virus diagnostic centers to examine the specimens submitted by hospital laboratories.

13. Introduction to Virology

13-1. We often tend to think of great epidemics of communicable diseases in terms of the distant past—the "plagues" of the Middle Ages that decimated entire populations. And we sometimes forget that many of the virus and rickettsial diseases that changed the course of world history over the centuries are still a threat to health, political stability, and economic welfare. It seems in order, therefore, to take a look at a few classic examples.

13-2. **Medical and Economic Significance.** Let's consider smallpox, for instance. Smallpox was prevalent in China and India before Christianity was founded. The virus was introduced into Western Europe early in the 16th century, and for the next 200 years the average annual mortality from this infection was nearly 20 percent of the population.[1] Jenner's discovery in 1798 of the principle of vaccination with cowpox virus provided a means of protection, yet smallpox epidemics exact a heavy toll of lives in Asia and Africa to this day.

13-3. Or take measles, a disease that is almost an inevitable accompaniment of childhood, even in the more advanced nations. Measles was recognized clinically 1900 years ago. Periodic epidemics have swept the world, causing uncounted fatalities among children and elderly susceptibles. History records that the line of succession to the French throne was altered when this disease invaded the court of Louis XIV in 1712 and struck down two immediate contenders. The measles virus remains unconquered despite promising research on new vaccines. Even the relatively mild Rubella, or so-called German measles, is estimated to have caused 20,000 birth defects in the 1964–65 outbreak that produced 2½ million cases in the United States.

13-4. Louse-borne typhus, a rickettsial infection, has influenced the outcome of many military operations over the years. In 1528 the French army besieging Naples saw imminent victory turned into defeat when typhus infected 30,000 soldiers and forced the French to withdraw from the city. The disintegration of Napoleon's army of 500,000 men in 1812 is believed to have been due in part to an epidemic of typhus during the retreat from Moscow. Long a problem in Eastern Europe and Asia, this disease swept the eastern front during World War I, reportedly causing 30 million cases and 3 million deaths among Russian military organizations and the civil populace. Notwithstanding the availability of modern pesticides, epidemic louse-borne typhus could explode again under crowded and unsanitary conditions which characterize the movement of troops and refugees in military campaigns.

---

[1] The elements of historical interest mentioned in this section were excerpted in part from the review by Schabel and in part from the text edited by Horsfall and Tamm which appear in the bibliography to this volume.

13-5. Yellow fever and influenza are among the many other virus diseases that could be mentioned from the standpoint of their historical impact on civilization. The first of these, yellow fever, appeared in Central America in 1648 and gave rise to epidemics in the Americas and parts of Europe until well into the 19th century. During the 200 years that elapsed before the role of the mosquito in yellow fever transmission was discovered, the disease-ravaged populations, brought industry and trade to a standstill in parts of the Western Hemisphere, and delayed the economic development of large geographical areas. The virus still lurks in the jungles of South and Central America. The presence of the natural mosquito vector in the U.S. affords an opportunity for the spread of yellow fever to this country if public health measures are ever relaxed.

13-6. Influenza was recognized historically in at least 30 major epidemics from the 16th to 20th centuries. The worldwide spread (pandemic) of this disease in 1918–19 affected 500 million people and caused 20 million deaths, either directly or through associated secondary infections. In the United States alone, 500,000 fatalities from influenza occurred in a population of 100 million. Many students will recall the outbreak of so-called "Asian flu" in 1957 which sickened 60 million Americans within a period of a few weeks.

13-7. The foregoing examples point up the fact that many virus diseases are still uncontrolled. Let's examine briefly the enormous health and economic consequences. It has been estimated that a virus infection of one type or another occurs from 4 to 6 times a year in each person in the U.S. Assuming a lifespan of 70 years, this means that the average person suffers from virus diseases during 10 percent of his lifetime.

13-8. From the economic standpoint, billions of manhours are lost annually (work absenteeism has reached 10 to 20 percent in recent flu outbreaks). In other forms of economic loss, consider the 123 million dollars required to eradicate foot-and-mouth disease in Mexico during the 1950's and the multimillion dollar annual loss to poultry producers in the U.S. caused by the avian lymphomatosis virus.[2] It should not be surprising, therefore, that scientific interest and research resources are being focused more and more on the virus and rickettsial agents of disease as they offer challenges of the present and future as well as the past.

13-9. **Classification.** You will recall that the bacteria we studied in Volumes 1 and 2 of this course belong to the class *Schizomycetes*, the "fission fungi." The division *Protophyta* (primordial plants) contains two additional classes besides the bacteria: the *Schizophyceae* (fission algae) and the *Microtatobiotes* (Greek, smallest living things). The virus and rickettsial organisms and related forms fall within the latter class. The seventh edition of *Bergey's Manual* shows the following taxonomic relationship among the microbes that we will mention in this chapter. Certain families and genera of lesser medical importance have been omitted:

- Class III - Microtatobiotes
  - Order I - Rickettsiales
    - Family I - Rickettsiaceae
      - Tribe I - Rickettsieae
        - Genus I - Rickettsia
        - Genus II - Coxiella
    - Family II - Chlamydiaceae
      - Genus I - Chlamydia
      - Genus V - Miyagawanella
  - Order II - Virales

13-10. The first family of the order *Rickettsiales* contains the *Rickettsia* species that cause such diseases as Rocky Mountain spotted fever, typhus, and trench fever. The genus *Coxiella* is represented by the Q-fever agent. In Bergey's second family, *Chlamydiaceae*, we find two genera, *Chlamydia* and *Miyagawanella*, whose formal names are seldom seen in current scientific texts. Once thought to be viruses, these micro-organisms have been referred to for many years as the Psittacosis-Lymphogranuloma venereum-Trachoma group.

13-11. More recent taxonomic schemes propose the designation "Bedsoniae" for the Psittacosis and Lymphogranuloma venereum agents of the genus *Miyagawanella*. The trachoma agent (genus *Chlamydia*) is then grouped separately with the pathogen of inclusion conjunctivitis. In this chapter we will use the more recent classification groupings, remembering that there is still considerable debate about the proper taxonomic position of those forms which fall somewhere between the rickettsiae and the true bacteria.

---

[2] Figures on the incidence of disease and the financial burden imposed by virus epidemics were extracted from the literature by Schabel and summarized in his paper which is listed in the bibliography.

13-12. Formal classification within the viruses had not been agreed upon at the time Bergey's seventh edition was printed, so neither families nor genera are found below the order *Virales*. Lacking conventional taxonomic guideposts, virologists have adopted an assortment of physical and chemical characteristics, epidemiological traits and morphological properties which enable us to fit most of the human viruses into the eight major groups listed below:

Ribonucleic Acid (RNA) Viruses	*Deoxyribonucleic Acid (DNA) Viruses*
Picornaviruses	Papovaviruses
Reoviruses	Adenoviruses
Arboviruses	Herpes viruses
Myxoviruses	Pox viruses

In this classification scheme, the chemical and physical makeup of the nucleic acid core (RNA or DNA, single or double strands, number of nucleotides), host-virus relationship in nature, and the type of virus replication (effects on host cell) are all important. So are details of morphology concerning the virus particles—size, presence or absence of an envelope, arrangement of protein molecules. We will describe some of the characteristic differences among the eight groups in our discussion of the nature and structure of viruses.

13-13. **The Nature of Viruses and Related Organisms.** The virus and rickettsial forms (to include related agents of uncertain classification, e.g., the Bedsoniae) are obligate parasites which invade the nucleus or cytoplasm of living cells. The parasite reproduces by redirecting the synthetic mechanisms of the host's cell. Disease in the host is a consequence of this rearrangement or modification of cellular functions. Viruses and rickettsiae survive in nature outside of living cells, but they are inert in the external environment. From this circumstance we derive the term "obligate" parasite. That is to say, a living, susceptible host cell is an absolute requirement for multiplication of the parasite. We will distinguish between viruses, rickettsiae, and related microbes in greater detail later in the chapter. At this point it is sufficient to recall that rickettsial forms are primarily parasites of the arthropods (ticks, mites, lice) whereas the viruses use plants, lower animals, humans and arthropods as hosts. Even bacteria have their virus parasites—the bacteriophages.

13-14. Awareness of the existence of viruses came late in the 19th century. Iwanowski's studies in 1892 showed that infective but microscopically invisible particles were passing through filters that retained bacterial cells. The fact that these filterable agents could not be grown by conventional culture techniques immediately set them apart from other microorganisms. For 30-odd years little more was learned about these invisible forms, although the refinement of laboratory equipment and animal experiments disclosed something of the size and pathogenicity of the particles. Stanley's isolation of the tobacco mosaic virus in crystalline form in 1935 shed first light on the chemistry of the intracellular parasites. Subsequently, there were three developments which opened the way for more extensive investigation. First, there was the discovery in the 1930's that viruses and rickettsiae will reproduce in fertile hen's eggs. The second was the development in 1949 of cell cultures (tissue cultures) in which the growth of viruses can be detected visually and their numbers counted. Lastly, the invention of the electron microscope permitted minute virus structures to be seen for the first time. Today we recognize the eight major groups of viruses previously listed, with more than 300 immunological types within these groups. Clinicians classify virus and rickettsial infections into 50 or more disease syndromes. The end is not in sight because new disease processes and hitherto unknown agents are being reported regularly from around the world.

13-15. In analyzing gross differences between viruses and other microbes such as the bacteria and fungi, several unique virus characteristics emerge:

- Simple chemical makeup.
- Small size.
- Absence of enzymes.
- Multiplication only in a susceptible cell.

Viruses consist of an inner core of nucleic acid (either ribonucleic or deoxyribonucleic acid, but never both) and an outer coat of protein. Certain viruses, in addition, display a membrane, or envelope, surrounding the protein coat. But compared to the structurally and physiologically complex bacteria and other single-celled organisms, the viruses have a relatively simple chemical composition.

13-16. In table 33 we find that viruses range in size from particles smaller than some of the large protein molecules (e.g., the polio virus) to bodies exceeding the dimensions of the smallest bacteria [about 150 to 300 millimicrons (mμ)]. In terms of *mass*, a large pox virus—say smallpox—is on the order of 1,000 times greater than the extremely small polio virus. This range is actually no more startling than the disparity in size found between more familiar forms such as the dog and the elephant. In general, however, the viruses are

TABLE 33

COMPARATIVE SIZE OF REPRESENTATIVE MICROORGANISMS

Microorganism	*Nature*	*Dimensions (millimicrons)*
Escherichia coli	bacterium	500 X 2000
Rickettsia burneti	Q fever rickettsia	250 X 1000
Psittacosis agent	Bedsonia group	350 X 500
Vaccinia virus	virus	250 X 300
Herpesvirus	virus	150
Influenza agent	virus	100
Adenovirus	virus	70
Polio virus	virus	30

smaller than other microbes with which you are familiar.

13-17. Unlike the chemically more complex rickettsiae and Bedsoniae (psittacosis-lymphogranuloma venereum group), the viruses have no enzymes. They depend upon the host cell to manufacture all of the ingredients needed for energy metabolism, replication of nuclear elements and production of the protein coat. We have already noted that viruses are inert outside the boundaries of a living cell and that they must infect a susceptible host cell in order for the aforementioned processes to take place.

13-18. In contrast to the viruses, the rickettsiae and Bedsoniae organisms represented in table 33 are large enough to be faintly visible in the ordinary light microscope as pleomorphic, coccobacillary forms. Considered to lie phylogenetically at some intermediate level between viruses and bacteria, they nevertheless require an intracellular environment for growth. Both RNA and DNA components are present, as are enzyme systems essential in energy-yielding reactions. As in the case of the true bacteria, these species reproduce by binary fission. The intracellular phase of the rickettsial and Bedsonia groups is poorly understood. Therefore, our description in the following pages of replication within the host cell will be limited to typical viruses whose life cycle has been more clearly defined. First however, we must study the structure of viruses as a basis for understanding how they reproduce.

13-19. **Virus Structure and Life Cycle.** Viruses can be said to have two stages in their life cycle: the intracellular phase of reproduction and the extracellular stage in which the virus exists inertly in the external environment. It is difficult to follow the morphological changes in a virus after the host cell has been penetrated, even with the most powerful electron microscope. Hence, most of our knowledge of structure is based on studies of the extracellular *virion,* the mature, infectious unit often referred to as the "elementary body," or simply as the "virus particle."

13-20. Like the bacteria we have studied, viruses differ somewhat in form, depending upon conditions of growth. But there is one basic design for all viruses—a protein shell or coat and its enclosed core of nucleic acid. The simplest forms of the virion structure are shown in figure 46. Notice that the protein coat, known as the *capsid,* exists in one of two forms: cubic or helical. Whether the form is *cubic* or *helical* is dictated by the arrangement of the protein molecules that make up the capsid. So all viruses are said to have only one kind of symmetry, either cubic or helical.

13-21. The cubic capsid is usually shown under electron microscopy to be a spherical single layer of protein composed of smaller morphological units, the *capsomeres.* The capsomeres, in turn, consist of groups or "bundles" of structural units. There is evidence that these structural units, which are chemical molecules too small to be seen, may comprise a single polypeptide chain or an aggregate of them. Helical capsids generally do not show capsomeres, as we note in figure 46. Their invisible structural units are not arranged into groups corresponding to the capsomeres of the cubic capsids.

13-22. The expression "cubic" comes from the arrangement of the protein molecules of the capsid into a polyhedron (a solid figure with several plane surfaces). In many cubic viruses, the capsid is an icosahedron (20-sided figure) inside which the nucleic acid is coiled, as we have demonstrated. The term "helical" indicates that protein molecules of the capsid are arranged in spiral form, with the inclosed nucleic acid core also stabilized in a spiral configuration. Viruses with helical symmetry are usually rod-shaped.

13-23. The virions we have just described represent the simplest forms of virus structure. Certain groups of viruses regularly display an "envelope" of lipoprotein surrounding the capsid. This envelope is believed to be built around the capsid as a mature virus particle passes through the host's cell membrane on its way to the outside environment. There are indications that some of the larger human viruses possess other well-differentiated anatomical structures, but these structures have not been fully characterized as to composition or function.

13-24. We can ascribe certain functions to the viral capsid and its inner core, but the role of the lipoprotein envelope, in those viruses that possess it, is still not clear. The capsid stabilizes the nucleic acid, presumably keeping this nuclear substance inert in the virion until a host cell is invaded. The capsid protects the core from destruction outside the host cell and probably also assists in the

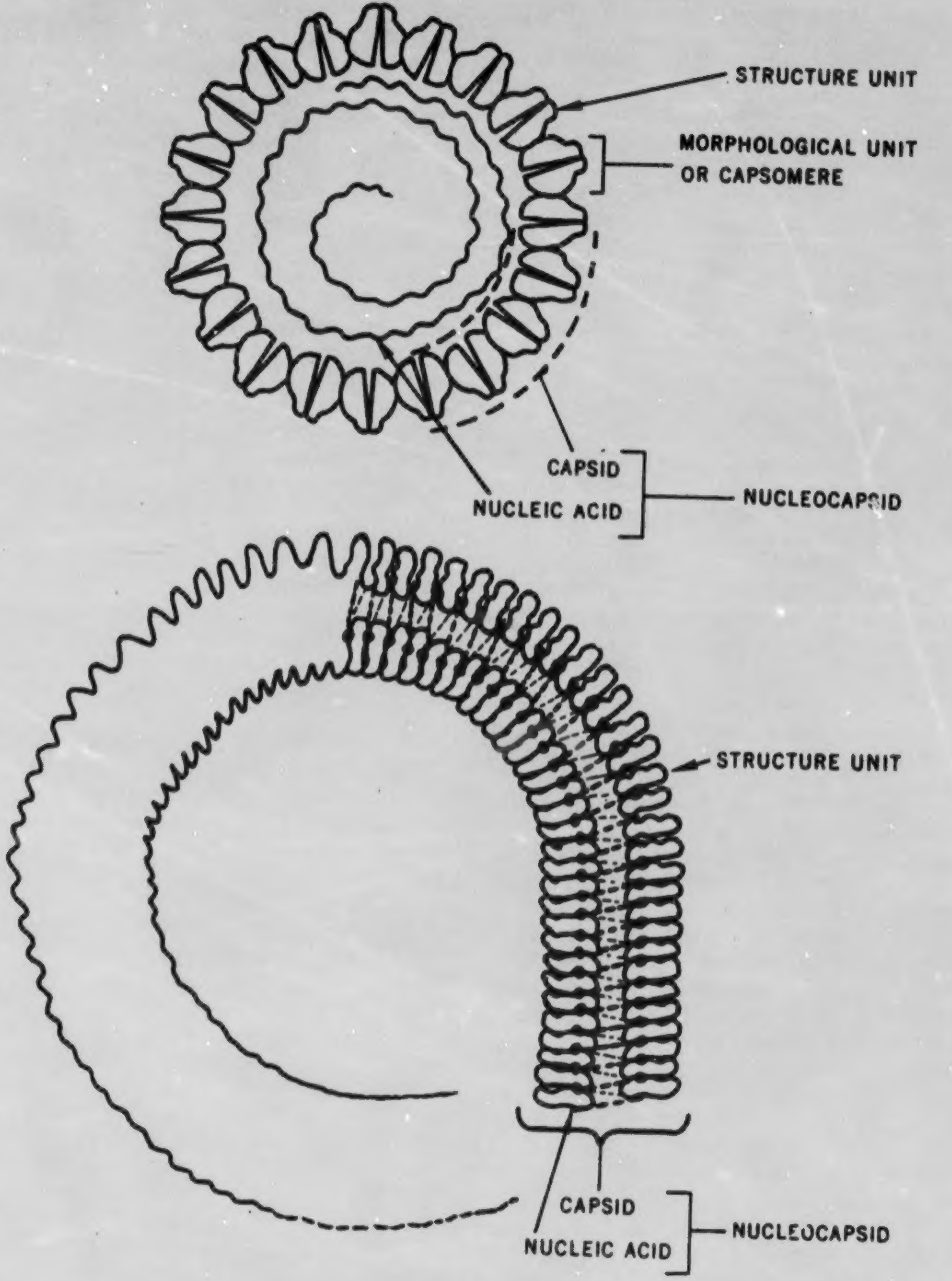

STRUCTURE UNIT
MORPHOLOGICAL UNIT OR CAPSOMERE
CAPSID
NUCLEIC ACID
NUCLEOCAPSID
STRUCTURE UNIT
CAPSID
NUCLEIC ACID
NUCLEOCAPSID

Figure 46. Cubic and helical verion structure.

attachment of the virion to the host's cell at the time of invasion. The viral protein is antigenic, i.e., stimulates the formation of immune substances (antibodies) in the host, and accounts for the immunological specificity of the virus types within the major taxonomic groups. The nucleic acid core carries the genetic coding information for virus replication; thus, the core corresponds to the genes in higher forms of life. We can look upon the mature virus particle (virion) as a vehicle for transporting infective nucleic acid, the virus core, between cells. Nucleic acids are long-chain polymers (multiples) of nucleotides. A nucleotide consists of either a purine or pyrimidine base linked to a 5-carbon sugar (a pentose) which, in turn, is linked to a molecule of phosphoric acid in the configuration we find in figure 47.

13-25. The life cycle of a typical virus can be broadly separated into the following phases:

- Virus enters a cell.
- Host cell synthesizes new virus components.
- Components are assembled into virus particles.
- Mature virus is released by the cell.

The virion attaches to and penetrates the cell (or is phagocytosed in some cases). At this point, the nucleic acid core separates from the protective capsid. The viral nucleic acid, acting as a template, or model, for its own multiplification, controls the synthesis by the host cell of new virus protein—both for necessary enzymes and for structural elements. Protein components and newly manufactured nucleic acid are than assembled into complete, infectious virus particles by the host cell according to the genetic code contained in the invading nucleic acid core. Mature virions are usually released by the host cell to complete the life cycle, but there are exceptions which will become apparent shortly.

13-26. The response of a host cell to virus infection can take three forms:

- Degeneration of the cell.
- Tumor formation.
- Steady state infection.

Degeneration can occur in either structure or function, ranging from complete lysis of the cell to less

NUCLEOTIDE STRUCTURE

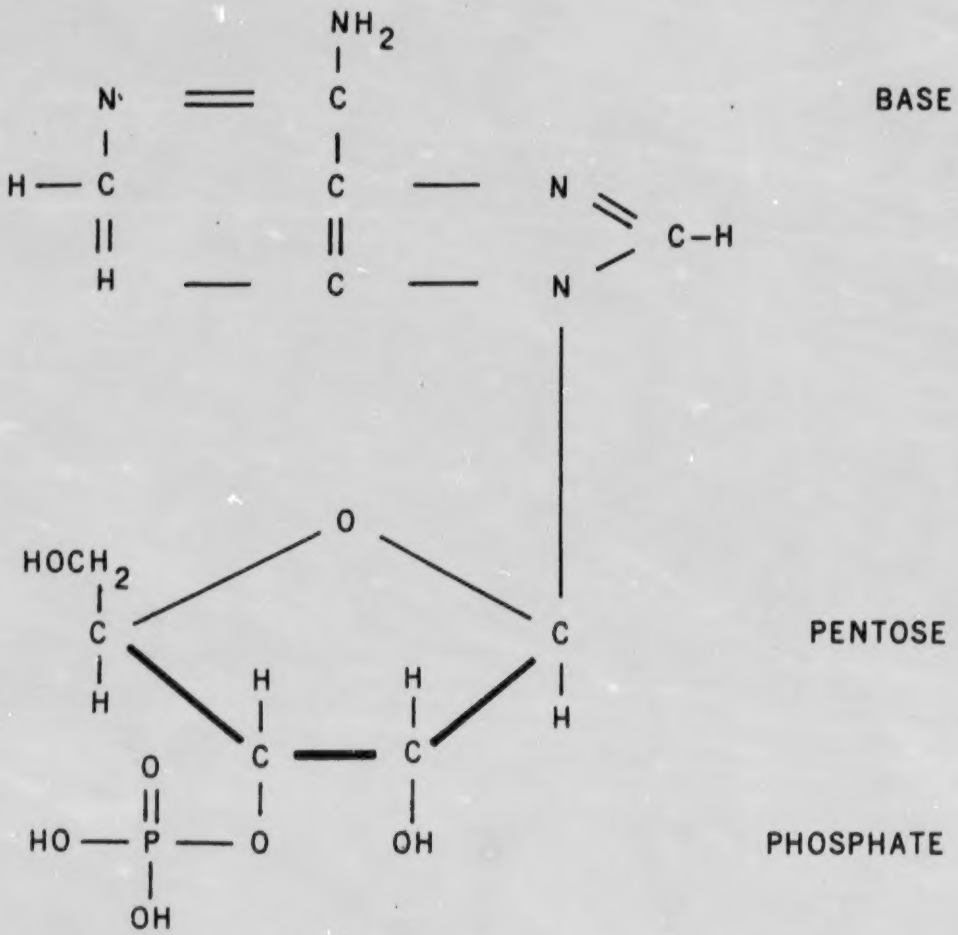
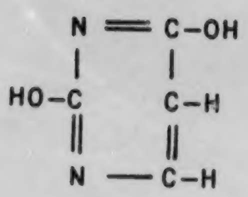
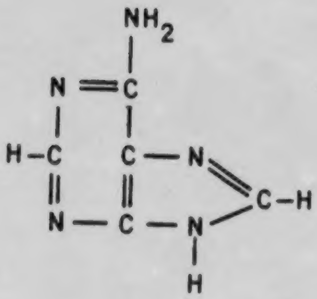


Figure 47A. Molecule of phosphoric Acid—nucleotide structure.

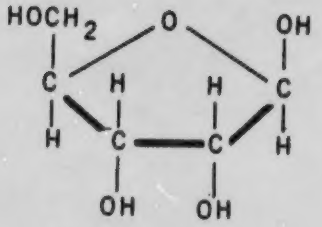
NUCLEOTIDE COMPONENTS



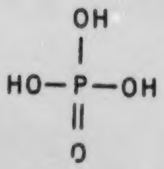
PYRIMIDINE BASE
(URACIL)



PURINE BASE
(ADENINE)



PENTOSE SUGAR
(RIBOSE)



$$HO-\overset{\overset{OH}{|}}{\underset{\underset{O}{\|}}{P}}-OH$$

PHOSPHORIC ACID
($H_3 PO_4$)

Figure 47B. Molecule of phosphoric Acid—nucleotide components.

pronounced cytopathic effects apparent only by measuring changes in physiological reactions. In either event, the effect is "cytocidal," i.e., it causes death of the cell. Transformation of a normal cell to the neoplastic state occurs in certain virus infections of the Papovavirus group. Papillomas of cattle and other animals, as well as human warts, illustrate this tumor-forming effect. In steady state infections, the host's cellular processes continue without serious disruption. The cells multiply and produce additional viruses through successive generations. It has been surmized that the viral nucleic acid may be able to synchronize its replication with the host cell's cycle of division and continue the steady state existence indefinitely. Evidence from research on bacteriophages suggests that the viral element may shift at any time from cytopathic to steady state and vice versa. The terms "latent infection" and "carrier state" have sometimes been applied to persistent steady state infections.

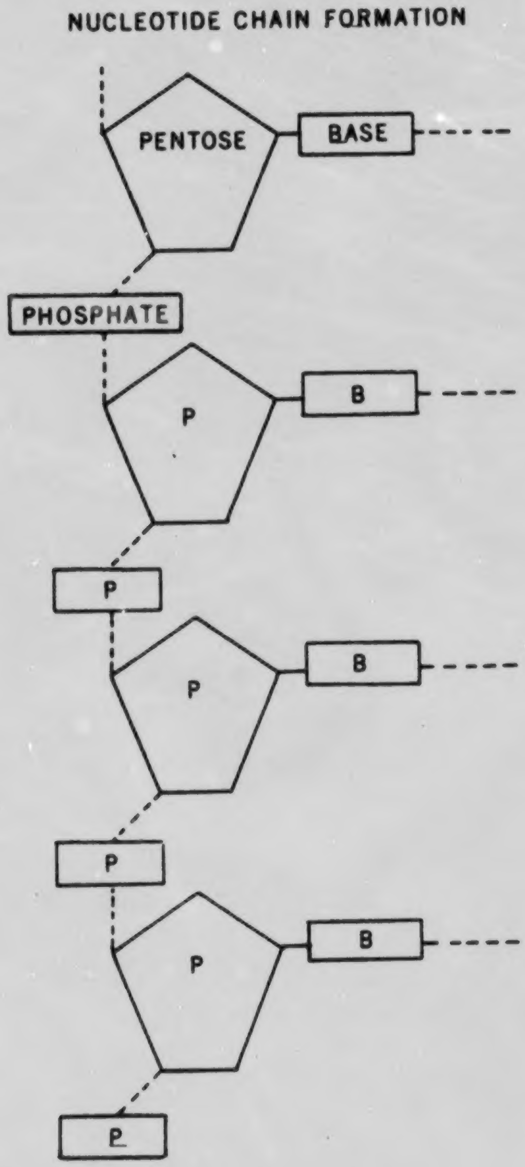


Figure 47C. Molecule of phosphoric acid—nucleotide chain formation.

14. The Major Groups of Pathogens

14-1. In this section we will cover some of the prominent features of each of the eight major virus groups and note certain of the pathological states that these viruses bring about. The rickettsiae and related forms will be treated similarly.

14-2. **The RNA Viruses.** There are four groups of viruses that contain ribonucleic acid (RNA), but not deoxyribonucleic acid (DNA) in their nucleic acid core. These groups, listed in table 34, are the Picorna viruses, the Reoviruses, the Arboviruses, and the Myxoviruses. Note that group distinctions can be made in most instances on the basis of inactivation by ether, symmetry of the capsid, and presence or absence of an envelope.

14-3. *Picornaviruses.* The name "Picornaviruses" implies extremely small size (pica-very small) and the presence of RNA. For the most part, these viruses range in size from 15 to 30 mμ; their resistance to inactivation by ether indicates the absence of a lipoprotein envelope; and they are protected from heat deactivation by positively charged ions (cations). We can subdivide this group into the following major categories:[3]

I. Human Picornaviruses
    a. Enteroviruses
        i. Polioviruses
        ii. Coxsackie viruses
        iii. Echoviruses
    b. Rhinoviruses
    c. Unclassified viruses
II Picornaviruses of lower animals

14-4. The *Enteroviruses* inhabit the human alimentary tract and give rise to infections ranging from nonspecific febrile illnesses to meningitis and paralytic diseases, as indicated in table 35. They can be recovered from throat washings and stool specimens, occasionally from blood, and rarely from urine and spinal fluid. The *Rhinoviruses* are found in nose and throat secretions, where they are reportedly associated with one-fourth to one-third of all acute upper respiratory infections in adults. These are the viruses most often responsible for the common cold.

---

[3] This grouping conforms to the classification scheme presented by Harsfall on page 14 of *Viral and Rickettsial Infections & Man* listed in the bibliography.

TABLE 34
CHARACTERISTICS OF MAJOR VIRUS GROUPS

Virus Group	Type Nucleic Acid DNA	 RNA	Ether Sensitive	Symmetry	Envelope
Picorna viruses	-	+	-	cubic	-
Reoviruses	-	+	-	cubic	-
Arboviruses	-	+	+	cubic	+
Myxoviruses	-	+	+	helical	+
Papova virus	+	-	-	cubic	-
Adenoviruses	+	-	-	cubic	-
Herpesviruses	+	-	+	cubic	+
Poxviruses	+	-	-	helical	+

14-5. The human Picornaviruses also include members whose exact relationship to the Enteroviruses and Rhinoviruses has not been fully established. These unclassified viruses have antigens in the protein coat (capsid) that are serologically identical to one or more antigens in other Picornaviruses, but otherwise little is known of their nature.

14-6. The Picornaviruses of lower animals include assorted Enteroviruses and Rhinoviruses whose existence was detected only after tissue culture techniques were developed. Additionally, some authorities place in this group the long-recognized, foot-and-mouth disease of cloven-hoofed animals and the Teschen disease virus of swine.

14-7. *Reoviruses.* This second major group of the RNA viruses comprises three immunologic types whose kinship is shown by size, similar cytopathic effects in tissue culture, and a common antigen detectable by complement-fixation. Table 34 reveals their similarity to the Picornaviruses. The term "reo" relates to the association of these viruses with illnesses of both the respiratory and enteric tracts. At this point, however, their importance as causative agents of disease is obscure. The Reoviruses can be isolated from feces and nose and throat secretions, but not from blood or urine.

14-8. *Arboviruses.* The third RNA group is composed of arthropod-borne viruses (hence the contraction Arboviruses) which multiply in man as well as in their primary blood-sucking hosts. About 150 antigenically related viruses (21 separate antigenic groups) are known, along with another 50 which lack the common antigen. Scrutiny of table 35 indicates that we usually speak taxonomically of the three subgroups: A, B, and C. Group A viruses, represented by the equine encephalitides, are transmitted only by mosquitoes. Group B diseases, such as yellow fever, dengue, and tick-borne encephalitis, are carried by mosquitoes and ticks, respectively. Group C viruses, few of which cause human infections, are parasites of mosquitoes, ticks, mites, the sandfly, and other species of biting flies. Among the ungrouped arbovirus diseases (not in A, B, C), we find less familiar names, such as Colorado Tick Fever, the hemorrhagic fevers of various Asia countries, Rift Valley Fever contracted by man from sheep, cows, and goats.

14-9. The diversity of morphological types in the Arbovirus group lends uncertainty to the question of the configuration of the capsid (cubic or helical) and the presence of an envelope in all cases, as found in table 34. In many instances the pathology of these diseases has not been well-defined. A viremia (viruses in the blood stream) is apparently essential to natural transmission by blood feeding arthropods, but secondary infections can arise from contact, as in the case of Rift Valley Fever of domestic animals.

14-10. *Myxoviruses.* The fourth major category of RNA viruses is the Myxoviruses, whose name denotes affinity for mucous membrane cells. These organisms fall into the two subgroups seen in table 35. Subgroup I contains the human influenza viruses as well as respiratory viruses of domestic animals, e.g., swine influenza, and fowl plague. Subgroup II holds the parainfluenza organisms, the mumps and measles viruses, and those agents causing the animal diseases, rinderpest, distemper, and Newcastle disease of fowl.

14-11. The Myxoviruses are chemically and structurally more complex than the other RNA viruses since they are thought to contain lipid and carbohydrates in addition to protein and RNA. The envelope reveals microscopically a pattern or radially oriented surface projections (spikes) whose function is so far unknown.

14-12. **The DNA Viruses.** The viruses whose nucleic acids are made up of DNA can be subdivided into the four major groups shown in the lower half of table 34. The first of these, the *Papovavirus group*, gains its name from a coupling of the first two letters of three viral entities: Papilloma virus of man and rabbits, Polyoma virus of

TABLE 35

DISEASES CAUSED BY VIRUSES

Virus Group	*Diseases Produced*
Picornaviruses	
Enteroviruses	Poliomyelitis, aseptic meningitis, upper respiratory infections, gastroenteritis.
Rhinoviruses	Common cold and upper respiratory infections.
Reoviruses	Associated with poorly defined infections of the respiratory and alimentary tracts.
Arboviruses	
Group A	Eastern, Western, Venezuelan encephalitis.
Group B	Yellow fever, dengue fever, tick-borne encephalitides.
Group C	Sandfly fever.
Myxoviruses	
Subgroup I	Influenza.
Subgroup II	Mumps, measles, distemper, rinderpest.
Papovaviruses	Papillomas (warts) of man and animals.
Adenoviruses	Upper respiratory infections, gastroenteritis, conjunctivitis.
Herpes viruses	Fever blisters, chicken pox, Monkey B virus.
Poxviruses	Smallpox, vaccinia, cowpox, molluscum contagiosum.

mice and vacuolating agent of monkeys. Viruses of this group are of great research interest because of their tendency to cause tumors in a wide variety of animals. Fortunately, only the papilloma virus that induces warts is known in humans. The characteristics of the Papovaviruses relative to other DNA virions are compared in the aforementioned table. Properites in addition to those shown must be used in differentiating the Papova and Adenoviruses.

14-13. The second group, the *Adenoviruses*, is composed of 45 or more serologically distinct types, which nevertheless possess an antigen shared by all members of the group. About 30 immunologic types have been isolated from humans and the remainder from lower animals. Three disease states are caused by the Adenoviruses: an acute respiratory infection, associated mainly with military recruits (virus types 4 and 7); a pharyngitis, caused by type 3; and conjunctival infections, caused by types 3 and 8. These viruses can be isolated from pharyngeal or ocular secretions and from stool specimens.

14-14. The *Herpesviruses*, the third major grouping of DNA viruses, are also described in table 34. Ether sensitivity in this case is unique among the DNA viruses. Most of us are familiar with herpes simplex, the lesions commonly referred to as "fever blisters" or "cold sores." Some others of the group are much more dangerous to human health. For instance, *Herpesvirus simiae* (B virus of monkeys) is said to be fatal in a high proportion of the cases in which the infection is transmitted to man by monkey bite.

14-15. *Herpesvirus hominis* infection can be manifested not only in the frequently encountered fever blister vesicle, but in disseminated disease of major body organs, including the eye, liver, brain and lungs in certain susceptible persons. Recent studies suggest that another virus of this group, the Herpes Zoster agent which causes a vesicular eruption on the skin, also causes Varicella, the childhood disease we remember as chickenpox.

14-16. The *Poxvirus* group of DNA viruses contains the largest and structurally most complex of all the virions. They approach in size (200 to 330 mμ) the Psittacosis-Lymphogranuloma venereum-Trachoma organisms (Bedsoniae and related forms) and the smaller bacteria. Note in table 34 that poxviruses, alone, among the DNA groups have a helical capsid. Some of the Poxviruses are listed in table 35, in terms of the diseases they produce. The smallpox virus (variola) is perhaps the one most familiar to us from the standpoint of its historical role as a killer of millions of people before the principle of vaccination was discovered.

14-17. The Poxviruses infect a number of lower animals as well as man. The word "pox" is derived from the typical lesions which these agents produce on the skin and mucous membranes. In this regard, the viruses can be isolated from vesicles and postules, or from mucous secretion and saliva. Most of our knowledge about the Poxviruses comes from studies on the vaccinia virus. This is a laboratory strain obtained originally from a cowpox infection and cultured for many years in laboratory animals to provide vaccine for smallpox immunization. Because of immunologic similarities among the Poxviruses, inoculation with the "vaccinia" strain confers protection against the smallpox virus.

TABLE 36

CLASSIFICATION OF RICKETTSIAE ACCORDING TO DISEASE

- I. Typhus Group
  - A. Epidemic typhus (louse-borne
  - B. Brill-Zinsser disease (recurring typhus)
  - C. Murine typhus (flea-borne)
- II. Spotted fevers
- III. Scrub typhus (Tsutsugamushi fever)
- IV. Q fever

14-18. The vaccinia virus is morphologically more elaborate than the simple viruses that have only an undifferentiated nucleic acid core protected by a protein capsid. Electron micrographs of thin sections of the virion suggest the presence within the core of a rectangular, biconcave disk containing triple strands of DNA. Two electron-dense masses lie adjacent to the disk. The concave disk as well as the double-layered virus membrane have well-defined ridges and spiculelike projections whose function is still unknown.

14-19. **The Rickettsiae.** We noted in an earlier discussion of taxonomic relationships that the rickettsiae fall somewhere between the viruses and the bacteria. Four distinct disease groups are recognized, as shown in table 36. The basis for the distinction lies in (1) the clinical features of the infections produced, (2) epidemiological considerations (the rickettsiae are transmitted by arthropods), and (3) immunological properties such as the possession of a common group antigen.

14-20. The intracellular parasites of these four groups are coccoid, rod-shaped organisms about 0.3μ (microns) in diameter. They contain both DNA and RNA, unlike the true viruses which possess only one or the other. Table 37 lists some of the better-known rickettsial diseases, the causative agents, and their natural vectors.

14-21. The epidemic form of typhus is carried from man to man by the body louse, as is trench fever, a disease prominent in the World War of 1914–1918. The recurring form of typhus, Brill-Zinsser disease, sometimes occurs several years after infection with *R. prowazeki* and does not involve a second contact with the vector. Flea-borne typhus is a natural disease of rats and mice. *R.*

mooseri infects man by way of the rat flea. The agents of the typhus fevers are recoverable from the blood of a patient and sometimes from bone marrow specimens.

14-22. The tick-borne rickettsioses include Rocky Mountain spotted fever, first recognized in the U.S., and several diseases more or less restricted to the Eastern Hemisphere. For example, boutonneuse fever is found in Africa and parts of India and Europe. Russia and Mongolia play host to North Asian tick-borne typhus, while Queensland tick typhus occurs in Australia. Rickettsial pox of the U.S. and Russia and Scrub typhus of the Asian countries are transmitted to man by mites parasitic on certain lower animals. Q-fever, originally isolated in Australia, is unique among the rickettsial diseases in that it is transmitted in nature by ticks, lice, mites, and parasitic flies. The infection occurs in a wide variety of domestic animals and birds. Man ordinarily contracts the illness through contact with meat and dairy animals or their excreta. The agent can be isolated from blood, sputum, urine and in some cases, spinal fluid.

14-23. **The Bedsoniae and Related Forms.** Taxonomists are still undecided on the proper classification of the microorganisms refered to usually as the Psittacosis-Lymphogranuloma venereum-Trachoma (PLT) group. Currently, the first two agents are classed together as "Bedsoniae." The trachoma agent is considered by many authorities to deserve separate classification, together with a similar form, the agent of inclusion conjunctivitis.

14-24. The PLT members all have certain characteristics that set them apart from the viruses, rickettsiae and bacteria. Morphologically, the PLT agents bear a close resemblance to each other. The organisms multiply in the cytoplasm of a host cell, where elementary bodies 0.2 to 0.3μ in size are found. Binary fission appears to be the mode of reproduction. Members of the PLT group are also related by possession of a common group antigen. Both RNA and DNA are present in the nuclear element. All are susceptible to chemotherapeutic drugs, unlike the viruses.

14-25. The form of the disease produced by these PLT organisms differs remarkably, however. The psittacosis agent can infect man and other animals, but outbreaks of disease are usually associated with birds. The psittacine birds (parrots, parakeets) provide a reservoir in nature. Pigeons, ducks, chickens, and turkeys fall victim to the infection and man contracts the disease via the respiratory route in dressing poultry, or by inhaling the agent in dust contaminated with droppings.

TABLE 37

REPRESENTATIVE RICKETTSIAE INFECTIONS

Disease	*Causative Agent*	*Vector*
Epidemic typhus	*Rickettsia prowazeki*	Louse
Brill-Zinsser disease	*R. prowazeki*	--
Murine typhus	*R. mooseri*	Rat flea
Trench fever	*R. quintana*	Louse
Rocky Mountain Spotted Fever	*R. rickettsi*	Tick
Tick-borne typhus fevers		
Boutonneuse fever	*R. conori*	Tick
North Asia tick-borne rickettsiosis	*R. siberica*	Tick
Queensland tick typhus	*R. Australis*	Tick
Rickettsial Pox	*R. akari*	Mite
Scrub typhus	*R. tsutsugamuski*	Mite
Q fever	*R. burneti* *(Coxiella burneti)*	Mite, tick, lice, biting flies

TABLE 38

SPECIMENS FOR ISOLATION OF VIRUSES AND RELATED FORMS

Respiratory	Influenza virus	Throat washings
	Adenovirus	Throat washings
	Rhinovirus	Throat washings
	Psittacosis agent	Throat washings, sputum
Central nervous system	Encephalitis viruses	Blood, brain
	Herpes virus	Blood, vesicle fluid
	Mumps virus	Blood, saliva
	Coxsackie virus	Throat washings, stool, spinal fluid
	Poliovirus	Stool, throat washing
Skin lesions	Small pox virus	Vesicle fluid, scrapings, blood
	Measles	Blood, vesicle fluid, scrapings
	Typhus rickettsia	Blood
	Q fever rickettsia	Blood
Venereal	Lymphogranuloma-venereum agent	Pus, bubo fluid
Enteric	Enteroviruses	Stool

The systemic illness is usually expressed as a pneumonialike syndrome. A pneumonitis also occurs in cattle and sheep after exposure to the psittacosis agent.

14-26. Lymphogranuloma venereum (also known as lymphogranuloma inguinale and venereal bubo) is a disease manifested by lesions of the genital organs. The agent is transmitted through sexual contact and can be isolated from pus or fluids taken from the surface lesion or bubo (swollen, infected lymph gland).

14-27. Trachoma is an ancient disease recognized over 3500 years ago as a major cause of blindness. The agent of this disorder was finally isolated in 1957. Trachoma and inclusion conjunctivitis organisms are grouped together because both invade the epithelium of the eye. Actually, the inclusion conjunctivitis pathogen causes an infection of the genitals. The eyes of new-born infants are contaminated at birth to set up an invasion of the conjunctiva.

15. Methods in Virology

15-1. We have learned what viruses are, and how their parasitic existence affects the host which they depend upon for all life processes. We will now consider the steps necessary to pinpoint the identity of a virus so that infection within the host can be controlled and the spread of the virus to other susceptible hosts curtailed.

15-2. **Collection and Shipment of Specimens.** The method for collecting and storing body fluids, tissues, and other clinical specimens may differ somewhat from one hospital to another. It depends to some extent upon time and distance factors in transporting specimens to a virus laboratory. Nevertheless, if we are attentive to certain basic considerations, the probability of correct diagnosis will be streagthened regardless of differences in technique. These basic considerations are:

- The proper specimen.
- Collection at the right time.
- Preservation during storage and shipment.
- An adequate clinical history.

15-3. The choice of a specimen is important, especially in trying to isolate a virus, because these infectious organisms are often localized in certain limited areas of the body or only in certain organs. In table 38 we have listed representative viruses and the specific, or in some cases, multiple specimens which can be expected to yield the agent. Timing is important, too, because during the course of the disease the infection may progress from a localized to a generalized state, or vice versa, so that a particular tissue or organ system may contain viruses at one point in time and not at another.

15-4. With respect to serum specimens for immunological assays, circulating antibodies are usually not formed in the early phase of an infection, as we see in figure 48. For that reason we collect paired serum samples—the first as soon as symptoms appear, the second 2 or 3 weeks later. If virus isolation techniques fail, then an elevated antibody titer in the second serum may indicate the identity of the causative virus. It follows, therefore, that time as a factor in specimen collection is equally applicable to virus isolation and serological procedures.

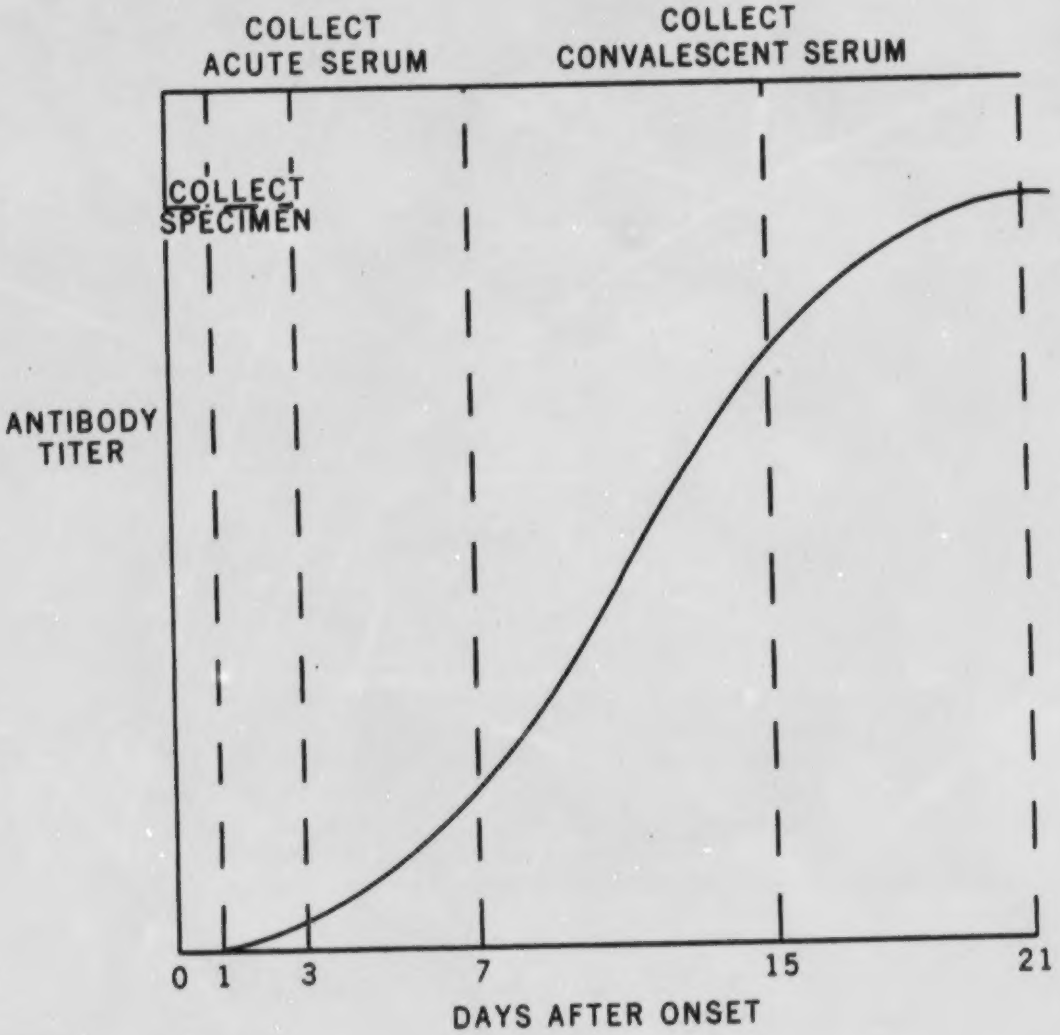


Figure 48. Time factor of specimen collection.

502

Request for viral and rickettsial diagnostic service

Requested by: ________________________ **Lab. No.** ____________

________________________ **(Location)** **Date Received** ____________

Patient's Name ________________ **Grade** __________ **AFSN** ________

Address ________________________ **Home Base** ________________

________________________ ________________ **(State)**

Age ______ **Race** ______ **Sex** ______ **Specialty** ______________

Vaccination against viral or rickettsial agents in past:

3 mos. ☐ **9 mos.** ☐ **12 mos.** ☐ **or year: 19**_____

Vaccine: **Influenza** ☐ **Smallpox** ☐ **Yellow fever** ☐ **Polio** ☐

Typhus ☐ **Spotted Fever** ☐ **Other** ________________________

Date of onset of illness ______________ **Date specimen sent** ______________

Specimen and date collected: **Whole blood** __________, __________, __________

Serum, acute __________ **Stool** __________

convalescent __________ **Throat washing** __________

Spinal fluid __________, __________, __________

Tissue (specific) __________: __________; __________: __________

Clinical data: **Febrile** ☐ **Afebrile** ☐ **Rash:** **Yes** ☐ **No** ☐

Respiratory tract involvement: **Yes** ☐ **No** ☐

Gastrointestinal tract involvement: **Yes** ☐ **No** ☐

Meningitis: **Yes** ☐ **No** ☐ **Paralysis:** **Yes** ☐ **No** ☐

Bulbar: **Yes** ☐ **No** ☐ **Fatal:** ______________________

Spinal fluid cell count ______________________

Contact: **Family** ☐ **Community** ☐ **Animal or birds** ☐ **Unknown** ☐

Therapy, if any: ____

Provisional clinical diagnosis: ________________________________

Tests required: ____

Figure 49. Typical history form.

531

15-5. We will mention specific techniques of specimen preservation a little later. But as a rule-of-thumb, viruses must be protected from drying (e.g., on throat swabs) and from the elevated temperatures found in a warm laboratory. Protection against drying can be accomplished by adding a suitable fluid. Refrigerating or freezing the specimen guards against lethal temperature affects. As we will see, there are also chemical additives which tend to prolong the viability of viruses.

15-6. Refer again to our aforementioned basic considerations in collecting specimens. The last of these, an adequate clinical history, is just as important as proper collection and protection of the virus. The diagnostic laboratory has available a number of biological systems for isolating infectious agents, and several serological tests can be applied. Some of these procedures work well with one group of viruses but less effectively with members of another group. The age of the patient, the clinical findings, the date of onset of disease, and related information furnished by the physician will aid the virus laboratory in selecting the proper procedures, or combination of test systems, to arrive at the correct identification of the causative agent. Figure 49 shows a typical history form to accompany a specimen.

15-7. There are individual preferences among virus laboratory directors with regard to the type of specimen that should be submitted to them for diagnosis. Preference is usually dictated by the kind of tests that a given laboratory can perform, i.e., the capability of that specific installation. Authorities in the field suggest that the following constitutes the *minimum* set of specimens to be collected from a patient at the time he first presents symptoms suggestive of a viral infection.

- A throat swab or nasopharyngeal washings.
- A stool specimen or rectal swab.
- A specimen of clotted blood.

In day-to-day practice, however, you should follow the guidelines for specimen collection, preservation, and shipment published by the laboratory which provides virus diagnostic services to your hospital.

15-8. *Collection procedures.* Having reviewed some of the underlying principles in collecting clinical materials for virus tests, we can now point out precise steps to take with each type of specimen and the reasons for these steps. *Blood* samples obtained by venipuncture (5 to 10 ml.) are collected aseptically within 3 days of onset of symptoms (the earlier the better) and again at 2 to 3 weeks, or before the patient leaves the hospital. Note in figure 48 that the "acute" phase serum may not show antibodies, whereas the "convalescent" phase specimen can be expected to have a detectable antibody titer. Don't forget, though, that a previous exposure to the virus, or past immunization with a vaccine, may have produced circulating antibodies which will be apparent serologically in the acute phase serum.

15-9. There is generally no set "diagnostic titer" of antibody to viruses; the increasing antibody level revealed by the convalescent serum (usually a fourfold increase or more in titer is meaningful) is the diagnostically significant information derived from serological testing. If no elevation is found when the two serum specimens are reacted with a known virus antigen, then infection with that given virus is ruled out. In practice, the sera are screened against a battery of several of the more common viruses, the composition of the antigen battery being determined by the patient's clinical record.

15-10. Be sure to separate the serum and the clot promptly and aseptically and place them under refrigeration, as we show in figure 50. Contamination of the specimen with fungus or bacterial growth can cause a breakdown of the serum components. The clot and a portion of the serum from the acute phase can be submitted at once for virus isolation. The remaining acute-phase serum should be stored frozen until the convalescent serum is obtained. Then the two are forwarded together to the virus laboratory for serological analysis. Ordinary refrigerator temperature (4° C.) will preserve most viruses for several days, but for prolonged storage use the laboratory freezer, or the ice-cube compartment of the refrigerator if no freezer is available. In either event, avoid repeated freezing and thawing of the specimen because some viruses are inactivated by this treatment.

15-11. *Stool* specimens for virus isolation may be collected as late as 5 to 7 days after symptoms occur. Since virus particles are usually present only in small numbers, 5 to 10 grams of feces should be collected from the bedpan or other receptacle. A rectal swab can be substituted for the formed stool, but virus isolation is apt to be less successful because of the scarcity of the virus in feces. The moistened swab should come in contact with fecal material, as indicated by staining. Store the swab in a tube containing 1 to 2 ml. of a sterile, balanced salt solution with a stabilizer such as 0.5 percent gelatin or bovine albumin. Nutrient broth, brain-heart-infusion broth and similar liquid bacterial culture media are also sometimes used as fluid carriers for swabs.

15-12. *Throat washings* are preferred to swabs of the nasopharyngeal region for isolating respiratory viruses. The patient is asked to cough 2 or 3 times; then he gargles about 15 ml. of buffered salt solution, nutrient broth, or similar harmless

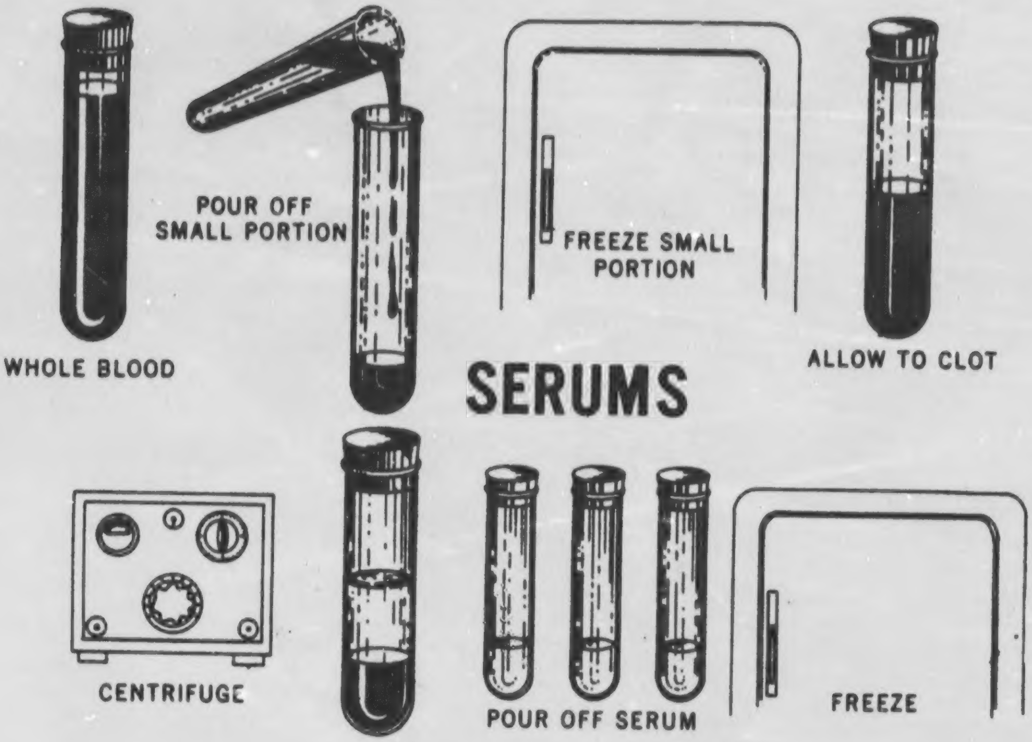


Figure 50. Aseptical handling of serum and clot.

fluid, and expectorates into a paper cup, as demonstrated in figure 51. He is asked to regargle the washings, after which the expectorated fluid is stored in a screw-capped sputum jar under refrigeration. Swabs of the nose and throat (infants and comatose patients) are stored in screw-capped tubes holding 1 to 2 ml. of one of the same fluids specified above for rectal swabs. This procedure for preserving viruses obtained by swabbing is generally applicable to other areas as well, i.e., conjunctivae, skin lesions.

15-13. *Miscellaneous* body fluids such as urine, cerebrospinal fluid, and pleural and pericardial infusions obtained aseptically by the physician are submitted in sterile tubes or jars. Several ml. of each fluid is needed for virus isolation attempts, and 10 to 50 ml. of urine is required. *Tissue* specimens collected aseptically at autopsy are handled similarly. These may include portions of lung, brain, heart muscle, liver, spleen, or kidney, among other possibilities.

15-14. *Techniques of preservation and shipment.* We pointed out earlier that low temperature is an effective means of maintaining the viability of most viruses; but, conversely, exposure of specimens to room temperature for only a few hours may prevent recovery of a virus. For a few hours storage prior to shipment, the refrigerator (4° C.) offers enough protection. In fact, if the specimens can be transported or mailed to reach the diagnostic laboratory within 24 hours of collection there is ordinarily no need to freeze them. An insulated carton containing wet ice or a can of commercial refrigerant (frozen for 24 to 48 hours) will maintain the desired low temperature for the period of transit. If no form of refrigeration is available, stools, tissue samples, and most other solid specimens can be protected to some extent by adding an equal volume of 50 percent glycerin. This substance has little effect in preserving liquids, however.

15-15. For other than the short period of storage and transport noted above, freezing (−70° C.) is our best method of protecting clinical specimens from deterioration. If you do not have a laboratory freezer which attains a temperature of −70° C., the ice cube compartment of the refrigerator is a convenient, temporary substitute. The specimens can also be sealed in glass ampules and frozen in a mixture of dry ice (solid CO_2) and alcohol, and subsequently kept frozen in dry ice. Shipping frozen materials to the virology laboratory by air or surface transportation requires additional dry ice and a sealed, insulated carton to retard loss of cooling capacity as the solid CO_2 passes into the gaseous state.

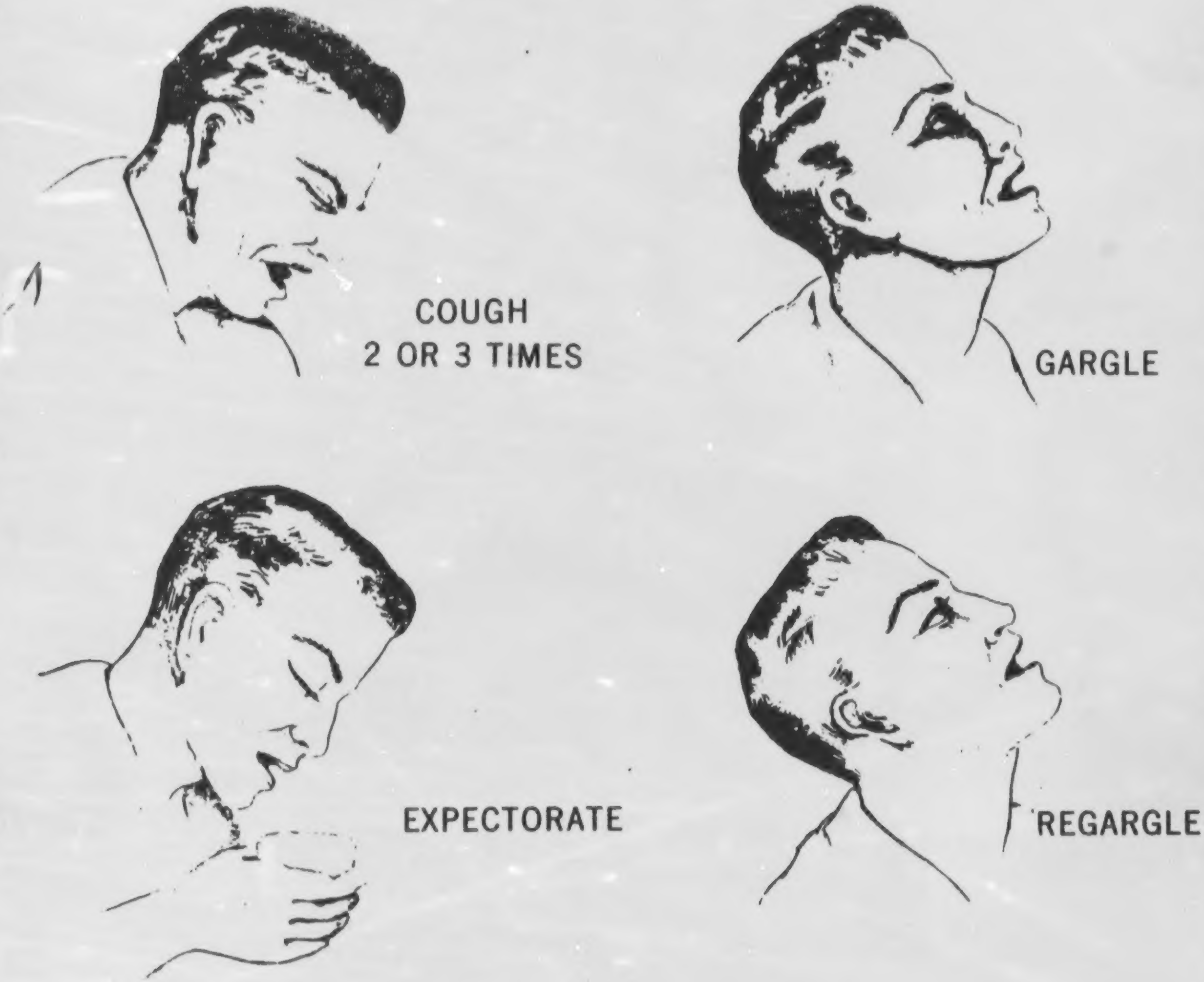

COUGH
2 OR 3 TIMES
GARGLE
EXPECTORATE
REGARGLE

Figure 51. Throat washings.

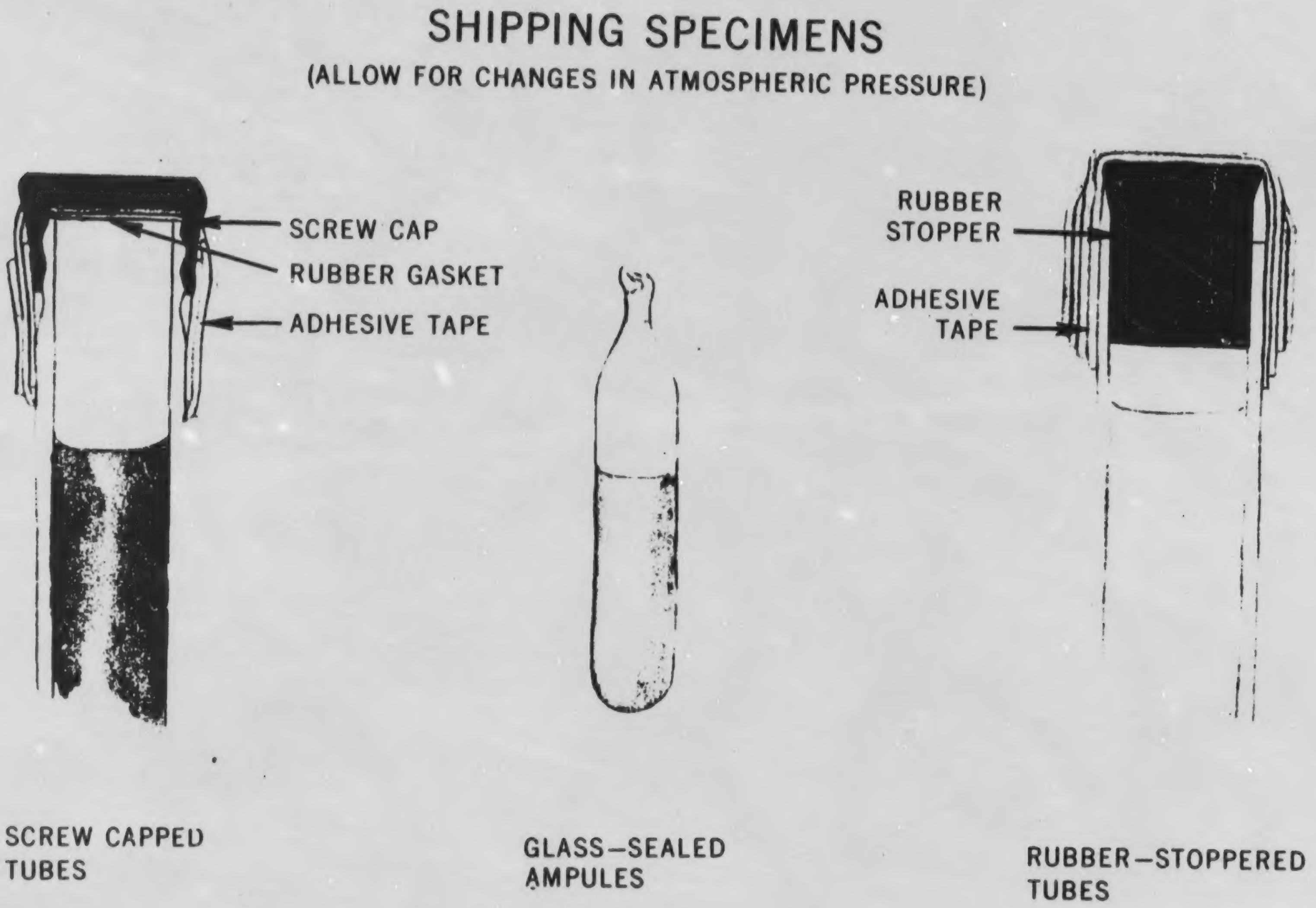

SHIPPING SPECIMENS
(ALLOW FOR CHANGES IN ATMOSPHERIC PRESSURE)
SCREW CAP
RUBBER GASKET
ADHESIVE TAPE
RUBBER STOPPER
ADHESIVE TAPE
SCREW CAPPED TUBES
GLASS–SEALED AMPULES
RUBBER–STOPPERED TUBES

Figure 52. Shipping precautions.

15-16. In transporting the clinical specimens to a reference laboratory, we must also consider other aspects besides low temperature, i.e., packaging for physical protection. The problems we most often encounter are leakage of fluids and breakage of the specimen container. Leaking occurs either because a tube or bottle was insecurely stoppered to begin with or a pressure change during transit by air breaks the seal. Tubes, jars, and bottles often break if they are dropped, and rough handling of the carton can cause the glass containers inside to crack or shatter if they come into violent contact through lack of sufficient packing material.

15-17. Ideally, clinical specimens should be protected by sealing them in thick glass ampules, as demonstrated in figure 52. Unaffected by pressure changes, these ampules also shield the specimen from chemical fumes that can inactivate a virus. Many laboratories have found screw-capped glass or durable plastic tubes and jars, as well as rubber-stoppered containers, satisfactory for shipping specimens if special precautions are taken with the closure. We show in figure 52 how a screw-capped tube can be fitted with a rubber gasket and then sealed with adhesive tape. As depicted in the figure, rubber stoppers can also be reinforced with a strong adhesive tape.

15-18. By wrapping individual containers with paper or cotton, we can decrease the opportunity for breakage from movement during shipment. Even greater protection is afforded if we wrap small groups of the tubes or jars and seal them inside tin cans. A number of outer containers are commercially available for protecting the ampules, tubes and jars which hold the clinical specimens. Fiberboard or foam-lined shipping cartons can be loaded with wet ice in plastic bags or solid CO_2, depending on whether the specimen is to be kept chilled or frozen. The various outer containers are designed to insulate the contents from environmental heat during transit periods of 24 hours to several days. The extent of protection depends upon such individual characteristics as size (ice capacity) and efficiency of the insulating material.

15-19. **Laboratory Diagnostic Procedures.** After a clinical specimen arrives at the reference laboratory, the virologist uses one or more of three broad approaches to diagnosis. In general, he uses a combination of procedures, or battery of tests, chosen from the following areas:

- Virus cultivation.
- Serological studies.
- Microscopic techniques.

In this course we will not cover detailed procedural steps in virus diagnosis. Rather, we will stress the principles underlying some of the more common tests and the reasons for performing them.

TABLE 39

DIAGNOSTIC METHODS AVAILABLE IN VIROLOGY

I. Virus Cultivation (Isolation)
  A. Laboratory animals
  B. Fertile hen's eggs
  C. Tissue culture
II. Serological Studies
  A. Complement fixation
  B. Neutralization test
  C. Hemagglutination inhibition
  D. Gel-diffusion techniques
III. Microscopy
  A. Fluorescent microscopy
  B. Electron microscopy
  C. Examination of cells for cytopathic changes

15-20. Recovery of the causative agent and its isolation in a living cell system is the purpose of virus culture procedures. In table 39 we note that three virus culture systems are available: animals, eggs, and cell cultures. The identity of a virus can often be established, at least tentatively, on the basis of pathological changes in a laboratory animal inoculated with a portion of the clinical specimen. The choice of the animal, e.g., guinea pig, mouse, depends upon what virus is suspected to be involved in the disease.

15-21. The growth of viruses and rickettsiae in fertile hen's eggs often gives characteristic changes in the embryo or neighboring tissues of the egg. The propagating microbes also furnish antigen (virus particles) for serological studies. Tissues cultures, the third of our common isolation systems, are prepared from human or animal cells that have become adapted to growth in nutrient fluids made up in the laboratory. If we inoculate suspected material into a tube or bottle containing tissue cells growing in a liquid medium, the viruses present in the specimen will invade the cells and multiply.

15-22. The virologist scans the tissue culture periodically for changes in the cells (cytopathic effects—CPE) characteristic of infection with certain groups of viruses. If the tissue cells are induced to grow in a thin sheet by imbedding them between layers of agar, the destruction of the cells in an area of virus propagation will yield a clear zone (plaque) whose size, shape, and appearance are also indicative of certain types of virus.

15-23. We have already learned that the measurement of circulating immune substances—antibodies—in acute and convalescent sera from a patient can often suggest the identity of an infecting virus. A higher antibody level (titer) in the convalescent serum than in the acute phase serum, when both serum samples are tested against a known virus antigen, gives us the clue. Table 39 shows that diagnostic laboratories are not restricted to only one form of antigen-antibody reaction. Serological procedures range from the time-honored complement-fixation test to newer methods of fluorescent microscopy. We will take a look at each of these later in this chapter.

15-24. The third main avenue in laboratory diagnosis open to the virologist (the others were virus cultivation and serological studies) takes advantage of microscopy to observe virus morphology or to visualize the effects of an agent in various cell preparations or tissue cultures. Table 39 lists some of the techniques. We search smears of cells for "inclusion bodies" or typical cytopathic effects. The electron microscope brings the smallest viruses in purified preparations into visual range for examination of shape, size, and other morphological features. The use of fluorescing dyes under ultraviolet light enables us to see microscopically the reaction between virus antigen in tissues and specially prepared antibody. We will return to fluorescent microscopy in our subsequent discussion of the principles behind representative diagnostic tests.

15-25. **Principles of Serological Testing.** The protein coat that surrounds the nucleic acid core of the viruses is usually antigenic, i.e., it causes a host animal to form immune substances (antibodies) in response to infection. Many differences exist in the *kind* of protein found in the virus coat, and antibodies tend to be specific for the one kind of protein (antigen) that gave rise to their production. We can take advantage of this phenomenon by setting up in the laboratory certain test systems that simulate the natural specificity of the antigen-antibody reaction. The serum portion of the blood contains the antibodies, hence, procedures using blood serum as a major ingredient are commonly referred to as "serological" tests. We can use such tests to identify a virus after it has been isolated, to detect a viral antigen in clinical specimens, and to demonstrate changes in serum antibody levels during infection.

15-26. Cross-reactions in serological tests are frequent, however, because an antibody may combine to a certain degree with antigens that are related chemically (but not identical to the protein that stimulated antibody formation in the first place. To cite an example, within the 200-odd arthropod-borne viruses about 150 contain common protein antigens that cause cross reactions in serological tests. For this reason, serological procedures must be carefully correlated with results of other diagnostic methods before the infectious agent can be accurately identified.

15-27. Most students in the clinical laboratory are familiar with routine "in vitro" forms of the antigen-antibody reaction. For instance, the visible clumping of a suspension of bacterial cells or red blood cells in the presence of an antiserum illustrates the agglutination test. Toxin-antitoxin reactions, in which a soluble rather than particulate antigen is used, typifies the conventional precipitin test. Viral and rickettsial antigens also form aggregates in the presence of specific antibodies, but the quantity of antigen needed to give an observable reaction is so large that some of our more common serological tests are impractical in the virology laboratory. As microtechniques are developed, and as means become available for preparing purified, concentrated antigens quickly and economically, agglutination, precipitation and flocculation methods will be more widely used. In the following paragraphs we will point out some of the serological procedures that are relied upon at present.

15-28. The *complement-fixation* (CF) procedure is widely used with the viruses and rickettsiae because only a small amount of antigen is required, and because the test has the proper sensitivity and specificity for routine use. Reagents to carry out the procedure on a sample of patient's serum include, in addition to the "unknown" serum, antigens from a "known" virus, sheep red blood cells (RBC), hemolysin (antibody to sheep RBC), and guinea pig complement.

15-29. Two systems are set up, a *test system* consisting of patient's serum, viral antigen, and complement; and an *indicator system* made up of sheep RBC and hemolysin. If the serum contains antibodies against the viral antigen, a combination of antigen, antibody and complement will take place. When complement is "fixed" in this *test* system, the reagent will be unavailable to combine with sheep RBC and hemolysin in the *indicator* system and lysis of the RBC will not occur. This is a "positive" CF test. Conversely, if no serum antibodies are present, the complement is free to combine with RBC and hemolysin. The resulting "negative" CF test is indicated by lysis of the sheep cells.

15-30. The *neutralization* test is suitable for identifying virus diseases, but not rickettsial infections. In principle, a virus infection causes the production of immune substances or "neutralizing" antibodies which will combine with virus particles in a test system, rendering the virus noninfectious. In practice, we can assay the neutralizing activity of a patient's serum by mixing aliquots of the serum with known viruses and incubating the individual mixtures in tissue culture, fertile eggs, or laboratory animals. An unneutralized virus will give cytopathic changes in tissue culture cells, lesions in eggs, death, or illness of the animal.

15-31. If, on the other hand, the patient's serum contains neutralizing antibodies, no change in the test system will be found because the virus has been inactivated. This latter situation represents a "positive" neutralization test. The principle is the same if we reverse the procedure and incubate unidentified virus antigen from a clinical specimen with individual sera containing neutralizing antibodies against known viruses. In other words, we can identify a virus by knowing the antibody constituents of specially prepared immune test sera.

15-32. Many viruses have the capacity to be adsorbed onto the red blood cells (RBC) of birds and mammals and to cause these cells to agglutinate. If antibodies to the virus are present, however, the virus particles are prevented from absorbing onto the RBC, and cell agglutination cannot take place. This is the basis for the *hemagglutination inhibition* test used to detect and assay viral antibody levels in patients' sera. In practice, we mix serum dilutions with a known virus antigen and allow the mixture to incubate. Group "O" RBC are added a short time later and observed for agglutination. If no antibodies are present to combine with the virus antigen, the RBC will be agglutinated at every serum dilution. If antibodies are present, however, their relative quantity (titer) can be determined by noting the highest dilution of serum that completely inhibits RBC agglutination.

15-33. *Gel diffusion* methods rely on the spread of antigens and antibodies through a solid, transparent menstrum such as agar. At the point where the components meet in optimum proportions within the agar, a visible precipitate forms to denote a positive reaction. Several variations in technique are used, but the "agar cell culture precipitation test" demonstrates the principle involved. We can grow tissue cells in a well (depression) carved in the surface of an agar plate and then infect the cells with an unknown virus isolate. We add immune sera of known antibody content to adjacent wells. After incubation, we search visually for lines of precipitated antigen-antibody between the wells. The corollary of this technique can be found in inoculating known viruses to the tissue cells and then adding patients' sera of unknown antibody content to adjacent wells. A positive precipitation reaction identifies the type of antibody, and thus indicates the virus to which a patient has been exposed.

15-34. One of the most promising methods of virus identification, *fluorescent microscopy*, embraces both immunologic and microscopic approaches. There are three points of information necessary for an understanding of the principle involved:

(1) Serum antibodies (gamma globulin fraction) can be coupled to certain fluorescent dyes (i.e., labeled) without losing affinity for the specific antigen that stimulated formation of these antibodies.

(2) The labeled antibodies (globulin plus dye) will combine with their specific viral antigen in infected tissue cells.

(3) The complex formed by antigen-antibody-fluorescent dye can be seen microscopically under ultraviolet illumination.

15-35. Fluorescent studies can be conducted in two ways: directly or indirectly. In the *direct* method, we smear a slide with tissue suspected of harboring a virus. Let's say that the laboratory has an array of antibody preparations, each specific for a certain known virus, and each previously conjugated (labeled) with a fluorescein dye. We add one of these preparations to the slide, incubate it a few minutes, rinse off the excess, and look at the slide under the microscope. If we find fluorescing areas in the tissue, it means that some of the labeled antibodies combined with the viral antigen and resisted the rinsing process (positive test). The absence of fluorescing areas or particles (negative test), indicates that no virus antigen specific for the antibody preparation was present in the tissue cells, and that the free antibodies were washed away during the rinse.

15-36. The direct method requires that the virologist keep on hand a large number of antibody preparations, each specific for one virus or group of viruses. It means, besides, that each of these antibody reagents must be conjugated (labeled) separately with the fluorescein dye, a time-consuming task.

15-37. The *indirect* method overcomes the disadvantages of having to label each different antibody preparation. Let's take a diagnostic situation in which we are trying to identify a suspected virus in an exudate received from a hospital. We prepare a slide of the smeared material, just as in the direct method. This time we add an unlabeled antiserum (not conjugated with a dye) prepared against a specifically known virus. It is unnecessary in this method to fractionate the serum to obtain the gamma globulin component. If our antiserum contains antibodies for the unknown virus in the smear, these antibodies will combine with antigen and resist being washed off the slide. Conversely, if no antibodies specific for that virus are present, the serum we added will be washed away completely.

15-38. The second step is to add to the washed smear an antiglobulin preparation labeled with a fluorescent dye. The reagent can be prepared by immunizing an animal with human gamma globulin, harvesting the animal's antibodies formed against the gamma globulin, and conjugating these antibodies with a dye. These labeled antihuman globulins will combine with the serum antibodies that we previously added to the slide (if they remained in the smear attached to antigen particles) and show fluorescence. If the antihuman globulins can be washed off the slide (no fluorescence appears after the washing process), it means that no serum antibodies against the virus, and hence, no virus particles were present in the smear. So, in the indirect method we are actually using an antibody prepared against another antibody to detect a virus.

Bibliography

Books

BREED, R. S., Murray, E.G.D., Smith, N.R. *Bergey's Manual of Determinative Bacteriology*, 7th Edition. Baltimore: Williams and Williams Co., 1957.

CONANT, Norman F., Smith, D.T., Baker, R.D., Callaway, J.L., Martin, D.S. *Manual of Clinical Mycology*. Philadelphia and London: W.B. Saunders Co., 1954.

EMMONS, Chester W., Binford, C.H., Utz, J.P. *Medical Mycology*. Philadelphia: Lea and Febiger, 1963.

FROBISHER, Martin. *Fundamentals of Microbiology*. Philadelphia and London: W.B. Saunders Co., 1962.

HARSFALL, F.L., Tamm, I. *Viral and Rickettsial Infections of Man*, 4th Edition. Philadelphia: J.B. Lippincott, 1965.

WILSON, J. Walter, Plunkett, O.A. *The Fungus Diseases of Man*. Berkeley and Los Angeles: University of California Press, 1965.

Periodicals

CHANOCK, R.M., Mufson, M.A., Johnson, K.M. "Comparative Biology and Ecology of Human Virus and Mycoplasma Respiratory Pathogens." *Progress in Medical Virology*, Vol 7, (1965), pp. 208–252.

MIDDLETON, P.J. "Virus Infections and the Diagnostic Laboratory." *New Zealand Medical Journal*, Vol 64, No. 390 (1965), pp. 61–71.

SCHABEL, F.M., Jr. "Virus Chemotherapy—Why and How." *Alabama Journal Medical Science*, Vol 2, No. 2 (1965), pp. 149–153.

WATERSON, A.P. "The Significance of Viral Structure." *Archiv fur die Gesamte Virusforschung*, Vol 15 (1965), pp. 275–300.

Department of the Air Force Publications

AFM 160–47, *Laboratory Procedures in Clinical Serology*, 1 March 1960.

AFM 160–53, *Laboratory Procedures in Clinical Mycology*, September 1963.

AFM 160–54, *Laboratory Procedures in Virology*, 23 August 1963.

Other Government Publications

KALTER, S.S., Hillis, W.D. *Procedures for Routine Laboratory Diagnosis of Virus and Rickettsial Diseases*, Publication #62–10, USAF School of Aerospace Medicine, November 1961.

Collection and Shipment of Virus Specimens, Illinois Department of Public Health, Division of Preventive Medicine, Weekly Report #3, 19 January 1968.

Laboratory Manual for Medical Mycology, Publication #994, Communicable Disease Center PHS, U.S. Dept. HEW, U.S. Government Printing Office, Washington D.C., 1963.

Glossary

Abstriction—Formation of asexual spores by the cutting off of portions of the sporophore through the growth of dividing partitions.

Acrotheca—A type of spore formation, characteristic of genus *Fonsecaea*, in which conidia are formed along the sides of irregular club-shaped conidiophores.

Aerobe—An organism whose growth and reproduction are favored by the presence of air of free atmospheric oxygen.

Anaerobe—An organism whose growth and reproduction are favored by the absence of air or free atmospheric oxygen.

Anthropophilic—Term applied to fungi that are considered to infect only man.

Arthrospore—Asexual spore formed by the disarticulation of the mycelium.

Ascospore—Sexual spore, characteristic of the class *Ascomycetes*, produced (union of two nuclei) in a sac structure known as an ascus.

Ascus—A specialized sac structure, characteristic of the class *Ascomycetes*, in which ascospores are produced.

Aseptate—Term describing absence of cross-walls in a hyphal filament or a spore.

Basidiospore—Sexual spore, characteristic of the class *Basidiomycetes*, produced (union of two nuclei) on a specialized club-shaped structure called a basidium.

Basidium—A specialized, club-shaped cell, characteristic of the class *Basidiomycetes*, on which exogenous basiospores are borne.

Blastospore—An asexual spore produced by a budding process, along the mycelium or from another blastospore.

Budding—An asexual reproductive process characteristic of unicellular fungi or spores involving the formation of lateral outgrowth from the parent cell that are pinched off to form new cells.

Cerebriform—Resembling the external convolutions and fissures of the brain.

Chlamydospore—Thick-walled resistant spore formed by the direct differentiation of the mycelium (concentration of protoplasm and nutrients).

Cladosporium—A type of spore formation characteristic of genera *Cladosporium* and *Fonsecaea*, in which conidia are formed in branched chains by conidiospores of various lengths.

Clavate—Club-shaped.

Cleistothecium—A structure, usually spherical, in which asci are formed.

Coenocytic—Term applied to a cell or an aseptate hypha containing numerous nuclei.

Columella—The prominent dome-shaped apex of a sporangiophore, characteristic of the class *Phycomycetes*.

Conidiophore—A specialized branch of hypha on which conidia are developed either singly or in groups.

Conidium—An asexual spore (one- to many-celled, and of many sizes and shapes) produced on a specialized structure known as a conidiophore.

Coremium—A sheaflike tuft of coindiophores.

Dematiaceous—Pigmented, dark in color.

Dimorphic—Term referring to those fungi which can grow and reproduce in *either* the mold form or the yeast form.

Echinulated—Spiny.

ECTOTHRIX—Arthrospores formed outside the infected hair shaft.

EN GRAPPE—In clumps on the ends of hyphae.

EN THYRSE—Individually along the sides of hyphae.

ENDOGENOUS—Derived from internal source.

ENDOSPORE—Spore formed within a special spore case.

ENDOTHRIX—Arthrospores formed *within* the infected hair shaft.

EXOGENOUS—Derived from external source.

FACULATIVE—Term referring to ability of an organism to grow and reproduce as an aerobe *or* an anaerobe.

FAVIC CHANDELIERS—Specialized hyphae that are curved, branched, and antlerlike in appearance; formed by certain dermatophytes, especially *Trichophyton schoenleinii.*

FAVIC-ENDOTHRIX—Hair infection resembling endothrix type; no arthrospores formed.

FUNGUS—A chlorophyll-less saprophyte or parasitic member of the plant kingdom, whose plant body is not differentiated into roots, stems and leaves. In most species the fundamental structural unit is mycelium.

FUSIFORM—Spindle-shaped.

GAMETANGIUM—A structure in which gametes are produced.

GAMETE—A sexual cell; especially a cell formed in a gametangium.

GEOPHILIC—Term applied to fungi whose natural habitat is in soil.

GERM TUBE—The tubelike process put out by a germinating spore that develops into the mycelium.

GLABROUS—Smooth, skinlike in texture.

GRANULOMATOUS—Composed of tumor or neoplasm made up of granulation tissue.

HABITAT—Natural place of growth of an organism.

HELICOID—Resembling a coil or helix.

HYALINE—Glassy and transparent.

HYPHA—One of the vegetative filaments composing the mycelium of mold fungi.

IMPERFECT FUNGI—Fungi that apparently lack the sexual means of reproduction and reproduce only asexually.

INTERCALARY—Said of spores produced between two hyphal segments.

KERION—A pustular infection of the hair follicles of the scalp characterized by a raised, boggy lesion.

MACROCONIDIUM—Large, often multicellular conidium.

MACROSCOPIC—Term referring to gross, cultural morphology that can be observed and studied with the naked eye.

MICROCONIDIUM—Small, single-celled conidium.

MICROSCOPIC—Term referring to minute morphology that can only be observed and studied under the lens of the microscope.

MOLD—Macroscopic: filamentous or mycelial form of fungus growth; microscopic: predominance of thread-like hyphae.

MONOMORPHIC—Term referring to those fungi which grow and reproduce in *only* the mold form or the yeast form.

MURIFORM—Term describing a conidium which possesses both transverse and longitudinal septations.

MYCELIUM—A mat of intertwined and branching threadlike hyphae.

MYCETOMA—Fungus tumor.

MYCOSIS—Fungus disease.

NODULAR ORGAN—A knot of close-knit hyphae considered to represent abortive attempts toward sexual reproduction.

ONYCHOMYCOSIS—A general term for a fungal infection of the nails.

OOSPORE—A sexual spore produced through the fusion of two unlike gametangia, found in the class *Phycomycetes*.

PECTINATE HYPHAE—Vegetative hyphal branches with unilateral digitate projections resembling teeth of a comb.

PERFECT FUNGI—Fungi that possess both the sexual and the asexual means of reproduction.

PERITHECIUM—A special closed structure in which asci are formed.

PHILOPHORA—A type of spore formation, characteristic of genus *Phialophora*, in which conidia are formed endogenously in flasklike conidiophores called phialids.

PLEOMORPHIC—Term describing the degenerative change of a fungus from a reproductive to a completely sterile colony. This process is irreversible and characteristic morphology is lost.

PSEUDOHYPHA—A chain of elongated budding cells that have failed to detach.

PYRIFORM—Pear-shaped.

RACQUET HYPHAE—Vegetative hyphae showing terminal swelling of segments suggesting a tennis racquet in shape.

RHIZOID—Term applied to radiating, rootlike hyphae, extending into the substrate.

SCLEROTIC—Hardened, thick-walled.

SEPTATE—Term describing presence of cross-walls in a hyphal filament or a spore.

SESSILE—Said of spores born directly on the hypha as indicated by the absence of a phore.

SPORANGIOPHORE—A specialized branch of hypha on which sporangia are developed.

SPORANGIUM—A closed structure within which asexual sporangiospores are produced by cleavage.

SPORE—Generally, the reproductive body of a fungus; occasionally, a resistant body for adverse environment.

SPOREPHORE—A general term for a spore-bearing structure.

STERIGMA—The singular of sterigmata.

STERIGMATA—Short or long projections (from the vesicle portion of a complex conidiophore) on which conidia are developed.

STOLON—A runner. A horizontal hypha which sprouts where it touches the substrate and forms rhizoids in the substrate.

SUPPURATIVE—Producing pus.

THALLOSPORE—Spores formed by a change in portion of the thallus.

THALLUS—A simple plant body; the vegetative part of the fungus plant as distinguished from the reproductive part.

TINEA—"Ringworm"; a prefix used with the infected area of the body (tinea capitis, tinea pedis) to indicate a cutaneous mucosis.

TRUNCATE—Having a side or an end cut squarely off.

TUBERCULATE—Having knobby projections.

VERMICULAR—Wormlike in shape or appearance.

VERRUCOSE—Warty in appearance.

YEAST—Macroscopic: pasty or mucoid form of fungus growth; microscopic: predominance of budding cells.

ZOOPHILIC—Term applied to fungi that infect lower animals as well as humans.

ZYGOSPORE—A thick-walled sexual spore produced through fusion of two similar gametangia; found in the class *Phycomycetes*.

☆U.S. GOVERNMENT PRINTING OFFICE: 1975—640-063 / 534

AUGAFB, AL (753240) 2300

90412 04 21

WORKBOOK

LABORATORY PROCEDURES IN CLINICAL MICOLOGY

SELF-TEACHING AIDS
CRE
VRE
SRG

This workbook places the materials you need *where* you need them while you are studying. In it, you will find the Study Reference Guide, the Chapter Review Exercises and their answers, and the Volume Review Exercise. You can easily compare textual references with chapter exercise items without flipping pages back and forth in your text. You will not misplace any one of these essential study materials. You will have a single reference pamphlet in the proper sequence for learning.

These devices in your workbook are autoinstructional aids. They take the place of the teacher who would be directing your progress if you were in a classroom. The workbook puts these self-teachers into one booklet. If you will follow the study plan given in "Your Key to Career Development," which is in your course packet, you will be leading yourself by easily learned steps to mastery of your text.

If you have any questions which you cannot answer by referring to "Your Key to Career Development" or your course material, use ECI Form 17, "Student Request for Assistance," identify yourself and your inquiry fully and send it to ECI.

Keep the rest of this workbook in your files. Do not return any other part of it to ECI.

EXTENSION COURSE INSTITUTE

Air University

TABLE OF CONTENTS

Study Reference Guide

Chapter Review Exercises

Answers to Chapter Review Exercises

Volume Review Exercise

ECI Form No. 17

STUDY REFERENCE GUIDE

1. *Use this Guide as a Study Aid.* It emphasizes all important study areas of this volume.

2. *Use the Guide as you complete the Volume Review Exercise and for Review after Feedback on the Results.* After each item number on your VRE is a three digit number in parenthesis. That number corresponds to the Guide Number in this Study Reference Guide which shows you where the answer to that VRE item can be found in the text. When answering the items in your VRE, refer to the areas in the text indicated by these Guide Numbers. The VRE results will be sent to you on a postcard which will list the *actual VRE items you missed.* Go to your VRE booklet and locate the Guide Number for each item missed. List these Guide Numbers. Then go back to your textbook and carefully review the areas covered by these Guide Numbers. Review the entire VRE again before you take the closed-book Course Examination.

3. *Use the Guide for Follow-up after you complete the Course Examination.* The CE results will be sent to you on a postcard, which will indicate "Satisfactory" or "Unsatisfactory" completion. The card will list *Guide Numbers* relating to the questions missed. Locate these numbers in the Guide and draw a line under the Guide Number, topic, and reference. Review these areas to insure your mastery of the course.

Guide Number	*Guide Numbers 400 through 424*
400	Introduction to Medical Mycology; Characteristics of the Fungi: General; Taxonomic Relationships, pages 1-3
401	Characteristics of the Fungi: Cellular Morphology, pages 3-6
402	Characteristics of the Fungi: Cultural Properties; Colony Morphology; Fungi as Agents of Disease, pages 6-8
403	Collection and Processing of Specimens, pages 8-11
404	Mycology Techniques, pages 11-14
405	Introduction to the Yeastlike Fungi and the Monomorphic Molds; The Pathogenic Yeastlike Fungi, pages 15-20
406	The Superficial Monomorphic Molds, pages 20-23
407	The Cutaneous Monomorphic Molds: General; Microsporum Audouinii; Microsporum Canis; Microsporum Gypseum, pages 23-27
408	The Cutaneous Monomorphic Molds: Genus Trichophyton, pages 27-30
409	The Cutaneous Monomorphic Molds: Epidermophyton Floccosum; Keratinomyces Ajelloi, pages 30-32
410	The Subcutaneous Monomorphic Molds, pages 32-35
411	The Systemic Monomorphic Molds: General; Clinical Aspects of Actinomycosis; Laboratory Diagnosis of Actinomyocosis, pages 35-40
412	The Systemic Monomorphic Molds: Nocardiosis, pages 40-42
413	Introduction to the Pathogenic Dimorphic Fungi; Subcutaneous Group, pages 43-45
414	Systemic Group: General; Coccidioides Immitis; Blastomyces Dermatitidis, pages 45-48
415	Systemic Group: Paracoccidioides Brasiliensis; Histoplasma Capsulatum, pages 48-52
416	Introduction to the Saprophytic Fungi; Saprophytes Commonly Encountered in Medical Mycology: Saprophytes with Characteristic Conidia, pages 53-59

Guide Number	
417	Saprophytes Commonly Encountered in Medical Mycology: Saprophytes with Characteristic Conidiophores; Saprophytes with Characteristic Sporangiophores; Saprophytes with Characteristic Thallus, pages 59-63
418	Saprophytes as the Etiological Agents of Rare Mycoses, pages 63-69
419	Virology: General; Medical and Economic Significance; Classification, pages 70-72
420	Virology: The Nature of Viruses and Related Organisms; Virus Structure and Life Cycle, pages 72-77
421	The Major Groups of Pathogens, pages 77-82
422	Methods in Virology: Collection and Shipment of Specimens, pages 82-89
423	Methods in Virology: Laboratory Diagnostic Procedures; Principles of Serological Testing, pages 89-92
424	Glossary, pages 94-96

CHAPTER REVIEW EXERCISES

The following exercises are study aids. Write your answers in pencil in the space provided after each exercise. Immediately after completing each set of exercises, check your responses against the answers for that set. Do not submit your answers to ECI for grading.

CHAPTER 1

Objective: To be able to describe the basis for classification of the fungi, the proper methods of collection and processing of specimens for either self examination or shipment, and the techniques commonly used in clinical mycology.

1. List the four classes of fungi. (1-2, 3)

2. Which of the classes of fungi contains most of the human pathogens? (1-3)

3. Why are the Deuteromycetes referred to as the fungi imperfecti? (1-3)

4. List the four microscopic criteria which often make a rapid accurate diagnosis possible. (1-4)

5. What is the twofold function of vegetative hyphae? (1-6)

6. List three important functions of the spore. (1-7)

7. What are the three varieties of the asexual spore? (1-8)

8. List the three types of thallospores. (1-8)

9. How does the endospore differ from the ectospore? (1-9)

10. Why are antibiotics incorporated into Sabouraud's dextrose agar? (1-11)

11. What are the three varieties of fungi based on colony appearance? Define each. (1-14-16)

12. What is another name for the agents of the cutaneous mycoses? (1-18)

13. Why must the laboratory worker exercise strict aseptic technique even when obtaining clinical material from skin lesions? (2-1)

14. Why must specimens of hair be obtained from the scalp even when no fluorescence is seen under ultraviolet light? (2-3)

15. List the techniques which should be routinely performed on sputum following direct examination and prior to culturing. (2-4)

16. List in proper sequence the steps routinely used when preparing specimens for shipment. (2-8)

17. Why is it inadvisable to ship such specimens as urine, bronchial washings, or biopsied tissue? (2-9)

18. In general, how does medical mycology differ from medical bacteriology regarding techniques used for identification? (2-9)

19. Why are cotton plugs preferable to screw-capped tubes for culturing fungi? (3-2)

20. How may the clearing effect of 10 percent KOH be hastened? (3-4)

21. What is the function of each ingredient in the stain lactophenol cotton blue? (3-5)

22. Why is the scotch tape wet mount technique preferred by some workers? (3-7)

23. What is the sole purpose of India ink when used to detect *Cryptococcus neoformans*? (3-8)

24. Why are larger tubes containing more media preferred for incubation at 37° C.? (3-9)

25. List the growth characteristics which should be noted when macroscopically examining a fungus culture. (3-11)

26. What advantage does the slide culture technique have over the teased or scotch tape wet mount? (3-14)

27. Why is a sterile coverslip placed over the cut in the media when inoculating a cut streak plate? (3-16)

CHAPTER 2

Objective: To show a knowledge of the clinical aspects and diagnostic laboratory techniques commonly employed in isolation and identification of the yeastlike fungi and the monomorphic molds.

1. Why are the yeastlike fungi which cause human disease considered opportunistic? (4-1)

2. List three conditions which permit the yeastlike fungi to become invasive or toxic. (4-1)

3. How do the *Cryptococci* differ from all other of the yeastlike fungi? (4-2)

4. Which of the yeastlike fungi never reproduces by means of simple budding? (4-2)

5. Why is it essential to isolate an unknown yeastlike fungi in its pure form? (4-3)

6. When using N HCL to obtain bacteria free cultures which tube of Sabouraud's dextrose broth is used for inoculation of a blood plate? (4-3)

7. Which technique described in Chapter 1 is used when the microscopic morphology is indicative of *Candida albicans*? (4-4)

8. List two reasons for a high incidence rate of Candidiasis in certain regions of the world. (4-5)

9. What is the name commonly applied to a *Candida albicans* infection of the oral mucous membranes? (4-6)

10. How does obesity result in candidiasis? (4-7)

11. Why do drug addicts often contract endocardial candidiasis? (4-8)

12. How would the technician preliminarily report a microscopic picture of budding yeast cells and mycelium pinched in the septations? (4-9)

13. What is probably the most common method used to identify *C. albicans*? (4-11)

14. What follow up tests should be used to identify *C. albicans* when chlamydospore production cannot be demonstrated? (4-11)

15. Describe a rapid method for identifying *C. albicans* using EMB media. (4-12)

16. Under what circumstances may a species of *Candida* other than *albicans* be considered as a possible disease agent? (4-13)

17. What is the probable reason for the low prevalence of infections with *Cryptococcus neoformans*? (4-14)

18. What is the most common portal of entry of infective spores of *C. neoformans*? (4-15)

19. What technique is routinely used to detect *C. neoformans* in clinical material? (4-16)

20. How is incubation temperature useful in differentiating *C. neoformans* from saprophytic cryptococci? (4-17)

21. What does a positive urease test indicate if the technician is attempting to identify a member of the yeast-like fungi? (4-18)

22. Why is the role of *Geotrichum candidum* in disease difficult to assess? (4-20)

23. What is an unusual characteristic of *G. candidum* regarding its colonial and microscopic morphology? (4-22)

24. How does a hair infected with *Trichosporon cutaneum* appear microscopically? (4-24)

25. What microscopic feature of *Pityrosporum ovale* readily distinguishes it from the other yeastlike fungi? (4-27)

26. What effect is produced when an individual with *Piedraia hortai* combs his hair? (5-2)

27. What symptoms are illicited by individuals infected with *Cladosporium werneckii*? (5-4)

28. How does *Malassezia furfur* differ culturally from the other superficial monomorphic molds? (5-7)

29. Why do the dermatophytes invade only the keratinized cutaneous tissue? (6-1)

30. To which class do the majority of dermatophytes belong? (6-2)

31. List three of the more common physiological tests used to identify members of the dermatophytes. (6-3)

32. List the three broad categories of dermatophytes based on their natural habitat. (6-4)

33. What is another name for a tinea unguium infection? (6-5)

34. What part do barber shops play in the transmission of *M. audouinii*? (6-6)

35. Define the term "kerion." (6-7)

36. When examining an early suspect case of tinea capitis for fluorescence under U.V. light, what added step should be taken to avoid a false negative result? (6-8)

37. How may *M. audouinii* be differentiated from other *Microsporum spp.* based on the use of rice grain media? (6-9)

38. Describe the typical microscopic characteristics of *M. audouinii*, when examined in wet mount from culture. (6-9)

39. With regard to the spontaneous healing of tinea infections due to *M. canis*, which form of the disease is of shorter duration? (6-10)

40. *a.* Why is it impossible to differentiate *M. audouinii* from *M. canis* on the basis of clinical material or symptoms?
    b. What procedure must be followed to identify these fungi? (6-11)

41. Describe the macroconidia of *M. canis* as observed in wet mount from culture. (6-12)

42. Where is *M. gypseum* found very frequently as a saprophyte? (6-13)

43. How do tinea infections of *M. gypseum* respond when viewed under a Wood's lamp? (6-14)

44. How would you describe the typical microscopic picture of *M. gypseum* when viewed in wet mount from culture? (6-15)

45. Which members of the dermatophytes are the most important causitive agents of tinea pedis and tinea unguium throughout the world? (6-16)

46. What is meant by "black dot" ringworm? (6-18)

47. How does the invasiveness of *T. rubrum* differ from *T. mentagraphytes* with regard to the "in vitro" hair culture test? (6-19)

48. What tissue normally attacked by the dermatophytes is not invaded by *E. floccosum*? (6-20)

49. Why are the morphological characteristics of the microconidia of *E. floccosum* of no diagnostic value? (6-21)

50. List two reasons why the ability to identify *K. ajelloi* is important. (6-22)

51. Describe the macroconidia of *K. ajelloi* in wet mount from culture. (6-24)

52. How are most cases of chromoblastomycosis contracted? (7-2)

53. List the three types of conidiophore formation which are demonstrated by the chromoblastomycotic fungi. (7-3)

54. How much of the body is usually involved in most cases of chromoblastomycosis? (7-4)

55. Describe the structures which make a rapid diagnosis of chromoblastomycosis possible in direct examination of clinical material. (7-6)

56. How does examination of wet mounts made from culture aid in specie identification of chromoblastomycotic agents? (7-7)

57. What does the term "mycetoma" mean? (7-8)

58. List two reasons for the greater incidence of maduromycosis in areas with hot, dry climates. (7-9)

59. How may the mycetomas caused by the maduromycotic fungi be microscopically differentiated from those caused by other fungi as the *Actinomyces spp.* or *Nacardia spp.*? (7-11)

60. List the gross characteristics which should be carefully noted concerning "grains" obtained from clinical material. (7-12)

61. Why is it inadvisable to use cycloheximide in the media when culturing for suspect maduromycotic agents? (7-13)

62. How are the members of the genera *Actinomycetes, Nocardia* and *Streptomyces* similar to bacteria? (8-2)

63. Why must the disease actinomycosis be considered of endogenous origin? (8-3)

64. What name is commonly applied to the tiny yellowish-white friable masses which are found in the purulent fluid in cases of actinomycosis? (8-4)

65. Define the term "lumpy jaw." (8-5)

66. Why is abdominal actinomycosis a definite threat in wounds of intestinal wall? (8-6)

67. List two important physiological requirements for primary isolation of *A. bovis* and *A. israelii*. (8-7)

68. Describe the appearance of *A. israelii* in thioglycollate broth. (8-8)

69. What is the microscopic appearance of a "sulfur granule" under high magnification? (8-9)

70. How may *Actinomyces naeslundii* be readily differentiated from *A. israelii* and *A. bovis*? (8-11)

71. Why is nocardiosis considered to be exogenous in origin? (8-12)

72. What is the general chain of events leading to the development of draining thoracic sinuses in cases of nocardiosis? (8-13)

73. In what respects do the mycetomas caused by the aerobic actinomycotic agents vary from those caused by *Actinomyces israelii*? (8-14)

74. What findings may be expected when grains from a case of nocardiosis are crushed, stained and microscopically examined? (8-15)

75. When culturing for *N. asteroides* or *N. brasiliensis* why is it necessary to inoculate the media prior to and following concentration? (8-16)

76. What difference is there in the growth rate between a *Nocardia spp.* and *M. tuberculosis* when incubated at 37° C. on media normally used to isolate M. tuberculosis? (8-16)

77. What two biochemical tests are routinely used for differentiation and identification of the *Nocardia spp.* and the *Streptomyces spp.*? (8-17)

CHAPTER 3

Objective: To show a knowledge of the clinical aspects and diagnostic laboratory techniques commonly employed in isolation and identification of the pathogenic dimorphic fungi.

1. When inoculated to suitable artificial substrates, what are the two forms of dimorphic fungi when incubated at 25° C. and 37° C. respectively? (Intro.-1)

2. In what way does the saprophytic form (mold form) of fungi gain access to the body? (Intro.-2)

3. Name the fungi classified as dimorphic. (Intro.-3)

4. Why is it important to diagnose infections of dimorphic fungi early? (9-1)

5. In what form are dimorphic fungi *not* transmissable? (9-1)

6. How can you help prevent laboratory infections when working with the dimorphic fungi? (9-2)

7. How does one normally become infected with the most common form of sporotrichosis? (9-4)

8. What characterizes the course of the disease sporotrichosis? (9-4)

9. Why are direct examinations of clinical material of little value in diagnosing sporotrichosis? (9-6)

10. List the patterns which describe the arrangement of conidia in cultures of *Sporotrichum schenckii.* (9-7)

11. Which phase of *S. schenckii* produces cells called "cigar bodies"? (9-8)

12. What must be done to confirm a diagnosis of *S. schenckii*? (9-9)

13. How are systemic fungi characterized? (10-1)

14. Name two forms of the disease coccidioidomycosis. (10-3, 4)

15. What is the name of the structure that contains endospores often found in clinical material in cases of coccidioidomycosis? (10-5)

16. How would you describe the mature arthrospores of *C. immitis?* (10-7)

17. Why should mycelial cultures of *C. immitis* be flooded with sterile normal saline prior to removing the cotton plug? (10-7)

18. What must be done to establish a confirmatory diagnosis of coccidioidomycosis? (10-8)

19. Name two immunological procedures used to help diagnose coccidioidomycosis. (10-9)

20. Give two additional names for Gilchrist's disease. (10-10)

21. List the clinical forms of blastomycosis. (10-11)

22. Which form of blastomycosis occurs most frequently? (10-12)

23. What cell produced in the tissue phase is characteristic of blastomycosis? (10-13)

24. How would the addition of cyclohexamide affect cultures of *B. dermatitidis* incubated at 37° C.? (10-14)

25. Name the tissue of the body for which *Paracoccidioides brasiliensis* has a special predilection. (10-16)

26. What part of the body serves as the primary focus of infection with *P. brasilienses*? (10-16)

27. Why is early diagnosis of infection with *P. brasiliensis* so important? (10-19)

28. How should clinical materials be cultured for *P. brasiliensis*? (10-19)

29. Briefly describe the two patterns of daughter cell budding in th[illegible]sue) phase of *P. brasiliensis.* (10-20)

30. What must be done to confirm cultural identification of *P. brasiliensis*? (10-21)

31. Why is the intratesticular injection with spores of *P. brasiliensis* useful? (10-22)

32. Which system of the body is preferred by the fungus *Histoplasma capsulatum*? (10-23)

33. How frequently does disseminated histoplasmosis occur? (10-24)

34. In what way is the treatment of clinical materials obtained from patients infected with histoplasmosis handled differently from clinical materials obtained from patients infected with blastomycosis or paracoccidioidomycosis? (10-25)

35. Within what types of cells are the yeastlike bodies of *Histoplasma capsulatum* found? (10-26)

36. Why should the tops of screw capped culture tubes be loosened occasionally after inoculation with clinical materials containing *Histoplasma capsulatum*? (10-27)

37. How are the characteristic macroconidia of *H. capsulatum* described? (10-28)

38. Under what circumstances may species of *Sepedonium* appear identical to those of *H. capsulatum*? (10-28)

39. How can nontuberculate *H. capsulatum* be differentiated with certainty from *Sepedonium* species? (10-28, 29)

40. Under what conditions may animal inoculation be a required procedure when diagnosing *H. capsulatum*? (10-30)

41. When should mice inoculated with spores of *H. capsulatum* be autopsied? (10-30)

42. What must be done after the animal is sacrificed? (10-30)

43. When is the precipitin test especially useful in testing for histoplasmosis? (10-32)

CHAPTER 4

Objective: To show a knowledge of the saprophytic fungi commonly encountered in medical mycology, and to be able to explain their role as the etiological agents of certain rare mycoses.

1. Why must the medical mycologist be able to distinguish the saprophytic fungi from the pathogenic forms? (Intro.-1)

2. List the eight genera of saprophytic fungi which have characteristic conidia. (11-2)

3. Why is the genus name "Curvularia" considered most appropriate? (11-7)

4. What is the probable reason for the genus name "Helminthosporium"? (11-9)

5. Describe the conidia produced by the members of the genus *Scopulariopsis.* (11-11)

6. What three structures make up the characteristic conidiophore in the genus *Aspergillus*? (11-14-17)

7. What is the name of the structure which generates spores in the genus *Penicillium*? (11-20)

8. List the four genera of saprophytes having a characteristic sporangiophore. (11-22)

9. What are two functions of the rhizoids found in *R. nigricans*? (11-25)

10. How are the microconidia of *Aureobasidium pullulans* produced? (11-29)

11. List two conditions which may predispose an individual to infection by a member of the saprophytic fungi. (12-1)

12. Which of the saprophytic fungi are responsible for the majority of human infections? (12-2)

13. Since *Aspergillus* species are such common laboratory contaminants, what criteria is necessary to implicate them in a disease process? (12-6)

14. Why are immunologic tests for *Aspergillus* of no value? (12-8)

15. List the four basic types of infections caused by the Phycomycetes. (12-10, 11)

16. Describe the fungal structures which may be observed in direct microscopic examination of clinical material from cases of phycomycosis. (12-12)

17. Why has the exact taxonomic position of *Rhinosporidium seeberi* never been established? (12-16)

18. How may the sporangia of *R. seeberi* be differentiated from those of *C. immitis*? (12-18)

19. How may *Cladosporium bantianum* be differentiated from *Cladosporium carrionii*? (12-22)

542

CHAPTER 5

Objective: To develop background information on viruses and related organisms sufficient for full understanding of the principles of specimen collection, preservation, and shipment to diagnostic laboratories.

1. The viruses fall within what taxonomic class of the division *Protophyta*? (13-9)

2. *Bergey's Manual* lists two genera of rickettsiae within the order *Rickettsiales*. What are these two genera? (13-10)

3. What are some of the factors considered in placing the viruses into eight major groups? (13-12)

4. Name the four RNA virus groups. (13-12)

5. Name the four classification groups of viruses that contain DNA in the nuclear core. (13-12)

6. Explain why viruses are called "obligate parasites." (13-13)

572

7. What scientist in 1892 discovered the existence of viruses, and what experiments led him to this discovery? (13-14)

8. Three major advances in laboratory technology opened the door to present-day knowledge of the viruses. What were these three advances? (13-14)

9. How did the invention of the electron microscope contribute to advances in our knowledge of viruses? (13-12, 14)

10. Describe several key characteristics of the viruses and rickettsiae that point to basic differences in their nature. (13-15-18)

11. What is the basic structural design found in all viruses? (13-20)

12. A viral capsid has one or the other of two kinds of symmetry: cubic or helical. Explain how the arrangement of protein molecules in the capsid influences this symmetry. (13-20-22)

13. List three probable functions of the capsid. (13-24)

14. What part of the virion serves as a template in directing the host cell to manufacture virus protein? (13-25)

15. The response of a host cell to virus infection can take three forms. What are they? (13-26)

16. The Picornaviruses comprise one of the four major groups of the RNA viruses. Within the Picornavirus group there is a subgroup whose members can be isolated from throat washings, stools, and sometimes from blood, although their natural habitat is thought to be the intestinal tract. Name this virus subgroup. (14-3, 4)

17. Among the RNA viruses we often find members of one of the major groups in stools and nasal or throat secretions. These viruses are associated with infections of the respiratory and enter tracts, although their part, if any, in *causing* these infections is obscure. Name this group of viruses. (14-7)

18. The arthropod-borne viruses (Arboviruses) consist mainly of three subgroups A, B, and C; name at least one disease from each subgroup and the type of vector involved in transmission. (14-8)

19. From what type of clinical specimens would you expect to isolate one of the pox viruses? (14-17)

20. Scrub typhus, Q fever, and epidemic (louse-borne) typhus are all caused by what major taxonomic group of viruslike organisms? (14-19)

21. How do the rickettsiae differ from the viruses with respect to their nucleic acid components? (14-20)

22. If your medical officer diagnoses an upper respiratory infection as psittacosis contracted from handling infected parakeets you would suspect the causative agent to belong to what major taxonomic group? (14-23-25)

23. Explain why the proper selection of clinical specimens is important in diagnosing virus diseases. (15-3)

24. How soon after onset of symptoms in a suspected virus infection should the convalescent phase serum be drawn? (15-4)

25. Select the correct word(s) in each sequence. As a rule (drying, high humidity) and (freezing temperatures, room temperatures) are harmful to viruses. (15-5)

26. As a general rule, in any suspected virus infection in which the causative agent is unknown, what 3 specimens should be collected? (15-7)

27. What is the lowest antibody titer found in convalescent sera that is considered significant when compared to an acute phase titer of 1-10? (15-9)

28. Why do we collect at least 5-10 grams of a stool specimen for virus isolation? (15-11)

546

29. If no form of refrigeration is available, what preservative could you use to protect stools, tissue samples, and other solid specimens during transit to the laboratory? (15-14)

30. Viruses can be grown in three types of living cell systems. What are these three systems? (15-20)

31. In virus diagnosis the technician uses one or more of three types or groups of procedures. Two of these procedures are virus cultivation and serological studies. What is the third procedure which may be used? (15-19, 24)

32. In addition to identifying isolated viruses, e.g., in tissue culture, and detecting viral antigens in clinical specimens, how do serological tests help us diagnose virus diseases? (15-25)

33. Explain the role of "hemolysin" in the complement-fixation reaction. (15-28, 29)

34. Describe the principle of the neutralization test. (15-30, 31)

576

55. The life cycle requires a stage of development in one of various arthropods. (8-26)
56. The dog and the cat. (8-28)
57. Because of their frequent close contact with infected cats and dogs as pets. (8-28)
58. They are of the same approximate length, but *D. caninum* usually has fewer proglottids. (8-29)
59. They resemble the shape of a cucumber or pumpkin seed. (8-29)
60. The eggs must be ingested by one of various insects, such as the dog louse or certain larval fleas, which serve as intermediate hosts. (8-30)

CHAPTER 4

1. Nematodo. (Intro.-2)
2. Intestinal and somatic or tissue roundworms. (Intro.-4)
3. *Trichinella.* (9-1)
4. The outer shell of the egg is sticky. (9-4)
5. Press the sticky side of clear scotch tape to the perianal folds of the patient and then stick the tape to a glass slide. (9-7)
6. *Trichuris trichiura* eggs are golden brown and barrel shaped. (9-10)
7. Because they are very resistant to drying and low temperatures. (9-13)
8. *Ascaris lumbricoides* eggs have a much thicker shell; and they contain an undivided cell mass, whereas hookworm eggs contain a zygote that is in the four or eight cell stage. (9-15)
9. *Necator americanus.* (9-18)
10. By penetrating the skin. (9-20)
11. One or two. (9-22)
12. Female. (9-26)
13. (1) The buccal canal of *Strongyloides* is very short, while that of the hookworm is longer and narrow; and (2) the genital primordium of *Strongyloides* is much larger than in the hookworm. (9-29)
14. It takes 7 to 14 days after exposure for most of the larvae to be filtered out in the muscle. (9-32)
15. Bentonite flocculation test and indirect hemagglutination test. (9-34)
16. In appropriate bloodfeeding insects. (10-2)
17. They circulate in peripheral blood mainly at night. (10-4)
18. Use 10 cc. of 2 percent formalin. (10-7)
19. The microfilariae of *Brugia malayi* have two cells in the tip of the tail which are not present in *Wuchereria bancrofti.* (10-9)
20. Mainly during the daytime. (10-11)
21. Black flies have chewing mouthparts; therefore, it is necessary for them to chew a hole through the skin to the capillary bed and then lap up the blood as it pools up in the hole. The microfilariae escape from the skin in the tissue fluids and are taken up with the blood. (10-13)
22. You will need to be able to differentiate them from the microfilariae of the more harmful species. (10-16)
23. They must be made deep enough to include dermal tissue. (10-17)

ANSWERS FOR CHAPTER REVIEW EXERCISES

CHAPTER 1

1. *a.* Phycomycetes.
   b. Ascomycetes.
   c. Basidiomycetes.
   d. Deuteromycetes.
   (1-2, 3)

2. The class *Deuteromycetes* sometimes called the fungi imperfecti. (1-3)

3. They are called the fungi imperfecti because they have no demonstrable means of sexual reproduction. (1-3)

4. *a.* Nature of the hypha.
   b. Type of spore.
   c. Type of spore bearing structure.
   d. Relationship of these structures to each other.
   (1-4)

5. It serves to anchor the fungus to the substratum, and it permits direct diffusion for procurement of nutrients. (1-6)

6. *a.* Duplication of the species.
   b. Dispersal to new substrata.
   c. Protection from adverse environmental conditions.
   (1-7)

7. *a.* Thallospore (body spore).
   b. Endospore.
   c. Ectospore.
   (1-8)

8. *a.* Arthrospores.
   b. Blastospores.
   c. Chlamydospores.
   (1-8)

9. The endospore is borne within a saclike membrane while the ectospore is borne free. (1-9)

10. They are used to inhibit the growth of bacteria and saprophytic fungi. (1-11)

11. The monomorphic yeasts, the monomorphic molds, and the dimorphic fungi. The monomorphic yeasts reproduce only by simple budding when incubated at either 25° C. or 37° C. The monomorphic molds reproduce only by spore germination when incubated at 25° C. or 37° C. The dimorphic fungi mulitply in the form of the monomorphic yeasts at 37° C. and in the form of the monomorphic molds at 25° C. (1-14-16)

12. The dermatophytes. (1-18)

13. The fungi causing serious systemic infections may disseminate producing cutaneous manifestations. (2-1)

14. Many tinea capitis infections caused by spores of the genus *Trichophyton* and *M. gypseum* will not fluoresce under ultraviolet light. (2-3)

15. *a.* Gram stain.
    b. Acid fast stain.
    c. Giemsa or Wright stain.
    d. India ink preparation.
    (2-4)

16. *a.* Clinical materials or isolates are placed in cotton plugged tubes, size 18 x 150 mm.
    b. Individual tubes are wrapped in cotton or paper and placed in a metal specimen container.
    c. Any free space left in the specimen container is stuffed with cotton or other packing material.
    d. The specimen container is capped and placed in a cardboard mailing carton.
    (2-8)
17. Because this type of specimen becomes rapidly overgrown with contaminants in transit, making the isolation of the pathogenic fungus improbable. (2-9)
18. In medical mycology greater emphasis is placed on differences in colony appearance and details of cell structure and arrangement, and less reliance on fermentation reactions and serological tests. (3-1)
19. Cotton plugs allow oxygen to enter the tube and permit the slant surface to remain dry, permitting better sporulation and pigment production. (3-2)
20. By gentle heating for a few seconds. (3-4)
21. *a.* Lactic acid serves as a preservative.
    b. Phenol serves as a killing agent.
    c. Cotton blue colors the structures making them more readily observable.
    (3-5)
22. It subjects the fungus to much less trauma thereby maintaining the structural relationships essential for accurate identification and is relatively simple. (3-7)
23. Its only purpose is to make the encapsulated organism more easily observable. (3-8)
24. The higher temperature tends to dry the media out more rapidly especially over extended period of incubation. (3-9)
25. *a.* Growth rate.
    b. Surface topography.
    c. Texture.
    d. Front and reverse pigmentation.
    (3-11)
26. The slide culture may be examined microscopically without disturbing the coverslip, thus structural relationships are maintained and mounting can be accomplished at the desired growth phase. (3-14)
27. To create microaerophilic conditions which are conducive to the formation of the fruiting structures of some yeasts. (3-16)

CHAPTER 2

1. Because the yeastlike fungi are frequently recovered from clinical specimens from individuals who display no adverse symptoms under normal conditions. (4-1)
2. *a.* Traumatic injury.
   b. Chronic debilitating and metabolic diseases.
   c. Increased use of antibiotics and steroids over extended time periods.
   (4-1)
3. All the *Cryptococci* have the ability to encapsulate. (4-2)
4. *Geotrichum candidum.* (4-2)
5. In order to perform accurate morphological studies and other tests that may be necessary. (4-3)
6. The acid tube which shows no bacterial growth after overnight incubation at 37° C. (4-3)
7. The cut streak technique. (4-4)

8. *a.* A high carbohydrate diet.
   b. Hot climates which result in profuse sweating.
   (4-5)
9. Thrush. (4-6)
10. When two skin surfaces are held together over long time periods, perspiration cannot evaporate and the urea breaks down into ammonia, resulting in a localized chemical initiation. (4-7)
11. Because they fail to adequately sterilize their needles and syringes. (4-8)
12. "Forms suggestive of *Candida* seen." (4-9)
13. The demonstration of chlamydospores using the cut streak technique. (4-11)
14. Sugar assimilation and fermentation tests. (4-11)
15. The suspect yeastlike fungi and a positive control are streaked on separate areas of an EMB plate which is incubated at 37° C. in a candle jar. *C. albicans* will produce germ tubes in from 4 to 12 hours. (4-12)
16. In cases where a species of *Candida* is repeatedly isolated from clinical material which is normally sterile, or when no other recognized pathogenic organism has been recovered from the clinical material. (4-13)
17. The failure to suspect its presence. (4-14)
18. Inhalation into the respiratory system. (4-15)
19. The India ink wet mount. (4-16)
20. Most saprophytic cryptococci are unable to grow at 37° C. (4-17)
21. This indicates that the yeastlike fungi is a member of the genus *Cryptococcus.* (4-18)
22. Because *G. candidum* usually occurs as a secondary invader. (4-20)
23. In its early growth phase *G. candidum* appears in the form of the other yeastlike fungi; however, as it matures, it assumes a fluffy mycelial character producing chains of arthrospores. (4-22)
24. You will see soft nodules ranging from white to light brown in color, loosely attached to the hair shaft. Athrospores and blastospores may also be seen. (4-24)
25. The bud is separated from the mother cell by a cross-wall. (4-27)
26. A clicking sound is produced. (5-2)
27. The organism causes the asymptomatic disease, tinea nigra. (5-4)
28. *Malassezia furfur* cannot be cultured on artificial media. (5-7)
29. Present indications are that other body tissues contain substances which inhibit the growth of these fungi. (6-1)
30. Class *Deuteromycetes.* (6-2)
31. *a.* Nutritional tests.
    b Optimal temperature requirements.
    c. Type of hair digestion "in vitro."
    (6-3)
32. *a.* Anthropophilic.
    b. Zoophilic.
    c. Geophilic.
    (6-4)
33. Ringworm infection of the nails. (6-5)

34. Use of unsanitary equipment has been found to be one of the most important modes of transmission of this fungus. (6-6)

35. A raised mass of tissue, supperating at numerous points. (6-7)

36. The suspect hair should be plucked and then examined under ultraviolet light since only the base of the hair is involved in early cases. (6-8)

37. *M. audouinii* grows very poorly on rice grains, while the other *Microsporum spp.* grow quite well. (6-9)

38. Microscopically, you would normally find pectinate or sterile mycelium with numerous chlamydospores. Some strains will form a few bizarre-shaped macroconidia and rare clavate microconidia borne sessile on the hyphae. (6-9)

39. The acute inflammatory type. (6-10)

40. *a.* They both cause similar symptoms and present a similar microscopic picture from clinical material.
    b. Cultural studies must be employed. (6-11)

41. The macroconidia are numerous, 8-15 celled, and spindle shaped. They often terminate in a distinct knob and have thick echinulated walls. (6-12)

42. In the soil. (6-13)

43. They fluoresce poorly or not at all. (6-14)

44. Numerous macroconidia, 3-9 celled, ellipsoid, shorter and broader than those of *M. canis*, with thinner echinulated walls. The microconidia are rare, clavate and usually borne sessile on the hyphae. (6-15)

45. Those belonging to the genus *Trichophyton.* (6-16)

46. An endothrix type infection caused by some members of the genus *Trichophyton* which causes the hair to break off close to or below the scalp surface creating a black, speckled appearance. (6-18)

47. *T. rubrum* will not perforate the hair while *T. mentagrophytes* is capable of causing wedge-shaped perforations. (6-19)

48. The hair. (6-20)

49. *E. floccosum* produces no microconidia. (6-21)

50. *a.* Data regarding the prevalence of dermatophytes in many regions of the world is scanty.
    b. The etiological agents of many ringworm infections go unreported and possible undiagnosed.
    (6-22)

51. The macroconidia are long and slender with parallel walls tapering at each end, and composed of 8-12 cells. The walls are wide and have a smooth surface. (6-24)

52. The causitive agents are probably saprophytic in nature and most cases result from embedding of the organism in wounds contaminated with soil or vegetative matter. (7-2)

53. Cladosporium, Phialophora, and Acrotheca. (7-3)

54. The disease tends to remain localized in the limb or area intially infected. (7-4)

55. The diagnostic picture seen in clinical material is one of small clusters of spherical dark brown, thick-walled cells that reproduce by equatorial splitting (not budding). (7-6)

56. The type or predominating type of sporulation in species exhibiting multiple type sporulation is useful in accurate identification. (7-7)

57. Fungus tumor. (7-8)

58. *a.* The causitive fungi flourishes under these conditions.
    b. The inhabitants are more likely to go barefoot.
    (7-9)

59. The mycetomas caused by the *Actinomyces spp.* and *Nocardia spp.* demonstrate mycelia of considerably less diameter (less than 1 micron) and elaborate no chlamydospores. (7-11)

60. *a.* Color.
    b. Texture.
    c. Shape.
    d. Size.
    (7-12)

61. Some of the fungi which cause maduromycosis are sensitive to this antibiotic. (7-13)

62. *a.* They cause suppurative tissue reactions.
    b. They are sensitive to many of the same antibiotics.
    (8-2)

63. The causitive agents of tne disease have never been isolated from any natural habitat and the fact that they are common inhabitants of the tonsillor crypts, gingiva and teeth of apparently normal individuals. (8-3)

64. Sulfur granules. (8-4)

65. "Lumpy jaw" is the term applied to cervicofacial actinomycosis in cattle and is caused by actinomyces bovis. (8-5)

66. Because *A. israelii* is often present asymptomatically in the human intestine. (8-6)

67. *a.* They both require an anaerobic or microaerophic environment.
    b. They both need an enriched medium.
    (8-7)

68. *A. israelii* produces discrete, labated, breadcrumb-type colonies about 1 cm. below the surface. (8-8)

69. High magnification shows the granule to be composed of intertwined delicate, branched filaments of 1 micron or less in diameter. (8-9)

70. By its ability to grow aerobically. (8-11)

71. The causitive fungi of nocardiosis are saprophytic in the soil. (8-12)

72. Infective spores are inhaled and produce a miliary type infection with a tendency for the lesions to penetrate the pleural wall, causing abscesses on the chest wall and rib involvement. These abscesses gradually extend outward to the skin surface. (8-13)

73. Different tissues of the body are affected and the grains which are formed will vary considerably in morphology and color, dependent on the species involved. (8-14)

74. Typical findings would be partially acid-fast, Gram-positive, thin- branching mycelium. (8-15)

75. It has been demonstrated that some strains of these organisms lose their viability when subjected to digestive agents. (8-16)

76. The *Nocardia spp.* will develop earlier than the tubercle organism. (8-16)

77. The casein hydrolysis test and the gelatin test. (8-17)

CHAPTER 3

1. At 25° C. these organisms will grow in a moldlike filamentous form; incubation of the same organism on the same substrate at 37° C. will result in the development of mucoid, bacterialike colonies of budding yeast cells. (Intro.-1)

2. From tissue damage or inhalation. (Intro.-2)

3. *Sporotrichum schenckii, Coccidioides immitis, Blastomyces dermatitidis, Paracoccidioides brasiliensis* and *Histoplasma capsulatum.* (Intro.-3)

4. The dimorphic fungi cause diseases that are incurable unless diagnosed early. (9-1)
5. These fungi are not transmissable in the parasitic or yeast form. (9-1)
6. Use a bacteriological safety hood, and exercise extreme caution and the best possible aseptic technique. (9-2)
7. The most common form of the disease normally results from implantation of the spores in the subcutaneous tissue during a penetrating injury from a thorn or splinter. (9-4)
8. The course of the disease is characterized by a chainlike lymphatic involvement with swelling of nodes and induration of the connecting lymphatics. (9-4)
9. In Gram-stained smears, it is very difficult to distinguish the few organisms which may be present from other tissue elements. (9-6)
10. "Sleevelike," and "flowerette." (9-7)
11. The tissue or parasitic phase. (9-8)
12. The yeastlike phase of the organism must be demonstrated. (9-9)
13. Fungi-causing deep-seated diseases involving one or more of the internal organs of the body. (10-1)
14. Primary pulmonary coccidioidomycosis and disseminated coccidioidomycosis. (10-3, 4)
15. A spherule. (10-5)
16. Barrel-shaped and tagged. (10-7)
17. To retard aerosol formation by arthrospores. (10-7)
18. Injection of test animals with mycelial growth and subsequent recovery of spherules produced in the guinea pig or mouse. (10-8)
19. The precipitin and complement-fixation tests. (10-9)
20. North American blastomycosis and blastomycosis. (10-10)
21. Primary cutaneous, primary pulmonary, chronic cutaneous, and disseminated. (10-11)
22. The chronic cutaneous form. (10-12)
23. Budding cells. (10-13)
24. The yeast phase of *B. dermatitidis* is sensitive to this antibiotic at 37° C. (10-14)
25. Mucus membranes. (10-16)
26. The lungs. (10-16)
27. Because the lesions simulate those caused by cutaneous leishmaniasis, yaws, and tuberculosis. (10-17)
28. By methods and materials recommended for the isolation of *B. dermatitidis.* (10-19)
29. *a.* Buds are quite small and vary only slightly in size ("steering-wheel" form).
    b. Buds vary appreciably in size (as much as 10 microns in length).
    (10-20)
30. Convert the yeastlike growth to the mycelial form, or the mycelial type growth to the tissue form. (10-21)
31. To establish the pathogenicity of a particular strain. (10-22)
32. The reticulo-endothelial system. (10-23)
33. A rare case progresses to disseminated histoplasmosis. (10-24)
34. The clinical material is best examined using Wright or Geimsa stained smears rather than unstained wet mounts. (10-25)

35. Mononuclear and occasionally polymorphonuclear cells (of the reticuloendothelial system). (10-26)

36. *H. capsulatum* is a strict aerobe. (10-27)

37. Large (7-25 microns in diameter), round to pyriform in shape, thick-walled, and usually having a tuberculated surface. (10-28)

38. Some strains of *H. capsulatum* do not produce tuberculate macroconidia. (10-28)

39. *H. capsulatum* can be converted to its yeast form at 37° C. (10-28, 29)

40. To accomplish conversion of those strains of *H. capsulatum* which will not convert to the yeast phase using the culture technique. (10-30)

41. At intervals of 16 to 42 days after injection. (10-30)

42. Infected tissues must be cultured on appropriate media at 37° C. in a further attempt to obtain yeast phase growth. (10-30)

43. In early diagnosis of the acute pulmonary form of the disease. (10-32)

CHAPTER 4

1. In order to prevent their being erroneously considered as the etiological agents of mycotic infection (Intro.-1)

2. *a.* Alternaria.
   b. Cephalosporium.
   c. Curvularia.
   d. Fusarium.
   e. Helminthosporium.
   f. Nigrospora.
   g. Scapulariopsis.
   h. Sepedonium.
   (11-2)

3. Because, typically, one side of the conidium shows greater curvature than the other. (11-7)

4. The wormlike shape of the conidiophore. (11-9)

5. They are lemon-shaped and echinulated developing in unbranching chains on the conidiophores; the apex is pointed with a truncate base. (11-11)

6. The stalk, the vesicle and sterigmata. (11-14-17)

7. The metula. (11-20)

8. *a.* Absidia.
   b. Mucor.
   c. Rhizopus.
   d. Syncephalastrum.
   (11-22)

9. Adherence to the substratum, and facilitation of assimilation of nutrient material. (11-25)

10. They may bud directly from the walls of the young hyphae or develop on short germ tubes elaborated by the older hyphal cells. (11-29)

11. *a.* People who have chronic debilitating disease.
    b. People whose normal immune response has been impaired.
    (12-1)

12. *a.* *Aspergillus spp.*
    b. Members of the class Phycomcetes.
    c. *Pencillium spp.*
    (12-2)

13. *a.* Repeated recovery from clinical materials.
    b. Absence of other pathogenic agents.
    c. Positive histologic findings.
    (12-6)

14. Because of the frequency of cross reactions and the lack of standardized reagents. (12-8)

15. *a.* cranial-facial.
    b. thoracic.
    c. abdominal-pelvic.
    d. dermal.
    (12-10, 11)

16. The structures include broad (6-15 microns in diameter) aseptate, branching hyphae. A rare septation may be observed. (12-12)

17. Because no one has been able to culture this organism. (12-16)

18. The mature sporangia of *R. seeberi* are of considerably greater size than those of *C. immitis.* Also the endospores of *R. seeberi* are lobated and stain darkly while those of *C. immitis* are nonlobated and only the endospore wall stains well. (12-18)

19. *C. bantianum* has a more rapid growth rate; also by the extreme length of its spore chains, the development of longer conidia and its ability to grow at temperature as high as 42° C. to 43° C. (12-22)

CHAPTER 5

1. The viruses belong to the class *Microtatobiotes,* a name derived from the Greek language meaning "smallest living things." The other two classes of the division *Protophyta* are the *Schizomycetes* (bacteria) and the *Schizophyceae* (the algae). (13-9)

2. The two genera are: *Rickettsia,* represented by the spotted and typhus fevers; and *Coxiella,* which contains the agent of Q fever. (13-10)

3. The physical and chemical properties of the viruses, the epidemiology of the diseases they produce, and differences in morphology provide the basis for classifying viruses into eight groups. The presence of RNA or DNA in the nucleic acid core, the effect of the parasite on the host (type of disease), and structural features of the virus particle are among the most important distinctions. (13-12)

4. The four groups of the RNA-containing viruses are Picornaviruses, Reoviruses, Arboviruses, and Myxoviruses. (13-12)

5. The DNA viruses are the Papovaviruses, Adenoviruses, Herpes viruses, and Pox viruses. (13-12)

6. Viruses are inert outside the cell of a susceptible host. Viruses can only reproduce if they are able to penetrate a cell and then redirect host cell functions so that new virus particles are formed. Because the intracellular environment of a host cell is necessary, we speak of the virus as an obligate parasite. (13-13)

7. Iwanowski noticed in 1892 that filters which removed bacteria from a fluid allowed other living forms to pass. These forms were invisible microscopically and they could not be grown on bacterial culture media. Nevertheless, the fact that these invisible agents gave rise to disease when injected into laboratory animals revealed their infectious nature. (13-14)

8. The growth of viruses in fertile eggs, the development of tissue culture procedures, and electron microscopy were major breakthroughs in research on viruses. (13-14)

9. The electron microscope permitted visual study of virus structures that cannot be seen with conventional light microscopes. Details of morphology, such as size, shape, arrangement of structural proteins, and presence or absence of an envelope, are useful in placing unknown viruses into one of the eight major taxonomic groups for identification. (13-12, 14)

10. Viruses contain either DNA or RNA, never both. Rickettsiae have both of these nucleic acids. Viruses depend upon the host cell for enzymes; many rickettsiae have preformed enzymes. Viruses reproduce by altering host cell functions to manufacture nucleic acid, proteins and other components of the virus particle. Rickettsiae reproduce by binary fission, similar to the true bacteria. In general the viruses are much smaller than the Rickettsiae and related organisms. (13-15-18)

11. Although viruses may differ somewhat in morphology, depending upon the conditions under which they are grown in the laboratory, the basic design common to all is a core of nucleic acid surrounded by a protein shell. (13-20)

12. In the cubic capsid the protein structural components are arranged into morphological groups or capsomeres, which form a spherical shell (capsid) around the nucleic acid core. This shell generally gives a polyhedral appearance, i.e. with the capsomeres arranged in several plane surfaces on the sphere. In the helical capsid the protein structural units are laid down in a spiral configuration around the nucleic acid core. This arrangement leads to an elongated body (capsid) whose external appearance is that of a short rod. (13-20-22)

13. The capsid stabilizes the nucleic acid core, protects the core when the virion is outside the cell, and probably assists in attachment of the virion to a cell of the host. (13-24)

14. The nucleic acid core of the virion contains the genetic code to be used by the host cell in manufacturing virus protein, nucleic acid, and all other components built into new virus particles. (13-25)

15. Cell degeneration, tumor formation, or steady state infections. (13-26)

16. The Enteroviruses, which include the polio and coxsackie and echoviruses. (14-3, 4)

17. Reoviruses. (14-7)

18. Group A - The encephalitci diseases carried by mosquitoes.
    Group B - Yellow fever (mosquito) and tick-borne encephalitides (ticks).
    Group C - Phlebotomus fever (sand fly).
    (14-8)

19. Pox viruses can usually be isolated from skin lesions (vesicles, pustules) and from saliva and mucous secretions at certain stages of the disease. (14-7)

20. Rickettsiae. (14-19)

21. The rickettsiae contain both DNA and RNA. Viruses contain one or the other, but not both RNA and DNA. (14-20)

22. The psittacosis agent belongs to the commonly labeled Psittacosis - Lymphogranuloma venereum - Trachoma group; more recently, it has been classed among the Bedsoniae. (14-23-25)

23. Many viruses tend to be localized in certain tissues at one point in the disease process, although they may be disseminated throughout the body at other specific times. Hence, knowledge of the type of specimen to collect in a suspected virus infection improves the chances for detection or isolation of the causative agent. (15-3)

24. Convalescent serum is collected 2 to 3 weeks after onset of symptoms or just before the patient leaves the hospital, if the specimen will not otherwise be obtainable. The time lapse after infection permits antibodies to the virus to be formed. These antibodies can be detected and measured as an aid in diagnosis. (15-4)

25. Drying and room temperatures are harmful to viruses, i.e., will inactivate them. (15-5)

26. Many authorities believe that the following comprise the minimum set of specimens: a throat swab or nasopharyngeal washings; a stool specimen or rectal swab; and a blood sample. (15-7)

27. A four-fold increase is usually significant, i.e., 1-40 versus 1-10 in the acute serum. (15-9)

28. Virus particles tend to be present in feces in relatively small numbers. A large portion of specimen (5-10 gram), when concentrated by the virologist, improves the probability of isolating a virus. (15-11)

29. An equal volume of 50-percent glycerin. (15-14)

30. Laboratory animals, cell cultures (tissue cultures), and fertile eggs. (15-20)

31. Microscopic studies of cytopathic effects or virus morphology. (15-19, 24)

32. Senological tests help diagnose viral diseases by demonstrating changes in serum antibody levels during infection. (15-25)

33. Hemolysin – an antibody to sheep red cells – is used as a component of the "indicator" system. The other components are sheep RBC and guinea pig complement. In the presence of complement, the hemolysin lyses the RBC, indicating a negative C-F reaction. (15-28, 29)

34. Virus infections give rise in the patient's serum to neutralizing antibodies. These antibodies will combine with their specific viruses in laboratory tests rendering the virus particles noninfective. Hence, we have a procedure for identifying an unknown virus (by knowing which antibodies are present), or for identifying a patient's antibodies (and thus his disease) if a known virus is used in the test. (15-30, 31)

35. A positive antigen-antibody reaction is revealed by a visible precipitate in a transparent medium at the point where antigen and antibody combine in optimum proportions. (15-33)

STOP- 1. MATCH ANSWER SHEET TO THIS EXERCISE NUMBER. 2. USE NUMBER 1 PENCIL.

90412 04 21

VOLUME REVIEW EXERCISE

Carefully read the following:

DO'S

1. Check the "course," "volume," and "form" numbers from the answer sheet address tab against the "VRE answer sheet identification number" in the righthand column of the shipping list. If numbers do not match, take action to return the answer sheet and the shipping list to ECI immediately with a note of explanation.

2. Note that numerical sequence on answer sheet alternates across from column to column.

3. Use only medium sharp # 1 black lead pencil for marking answer sheet.

4. Circle the correct answer in this test booklet. After you are sure of your answers, transfer them to the answer sheet. If you *have* to change an answer on the answer sheet, be sure that the erasure is complete. Use a clean eraser. But try to avoid any erasure on the answer sheet if at all possible.

5. Take action to return entire answer sheet to ECI.

6. Keep Volume Review Exercise booklet for review and reference.

7. If *mandatorily* enrolled student, process questions or comments through your unit trainer or OJT supervisor.
   If *voluntarily* enrolled student, send questions or comments to ECI on ECI Form 17.

DON'T

1. Don't use answer sheets other than one furnished specifically for each review exercise.

2. Don't mark on the answer sheet except to fill in marking blocks. Double marks or excessive markings which overflow marking blocks will register as errors.

3. Don't fold, spindle, staple, tape, or mutilate the answer sheet.

4. Don't use ink or any marking other than with a # 1 black lead pencil.

Note: The 3-digit number in parenthesis immediately following each item number in this Volume Review Exercise represents a Guide Number in the Study Reference Guide which in turn indicates the area of the text where the answer to that item can be found. For proper use of these Guide Numbers in assisting you with your Volume Review Exercise, read carefully the instructions in the heading of the Study Reference Guide.

Multiple Choice

559

Chapter 1

1. (400) The class *Phycomycetes* is characterized by the development of

a. broad septate hyphae.	c. ectospores.
b. broad aseptate hyphae.	d. thallospores.

2. (400) The members of the class *Deuteromycetes* are called the "fungi imperfecti" because they

   a. lack a demonstrable means of sexual reproduction.
   b. cause the most serious diseases in man.
   c. do not exist in perfect yeast form.
   d. reproduce only sexually.

3. (401) A mass of hyphae is correctly referred to as a

a. perithecium.	c. basidium.
b. mycelium.	d. sporangium.

4. (401) In direct microscopic examination of fungal growth, the feature which is of *least* diagnostic value is the

a. type of hypha.	c. hypha and spore-bearing structure relationship.
b. type of spore and spore-bearing structure.	d. spore-hyphal relationship.

5. (400) The most important function of the spore is

   a. protection from adverse environmental conditions.
   b. duplication of the species.
   c. dispersal of the fungus.
   d. incorporation of nutrient material.

6. (401) Macroconidia are characteristically

a. borne in a saclike membrane.	c. large and multicellular.
b. small and multicellular.	d. large and unicellular.

7. (402) The most serious mycoses are caused by the fungi described as

a. subcutaneous.	c. cutaneous.
b. superficial.	d. systemic.

8. (403) A patient with a tinea capitis infection

   a. always exhibits fluorescence under a Wood's lamp.
   b. never exhibits fluorescence under a Wood's lamp.
   c. may exhibit fluorescence under a Wood's lamp.
   d. need not be re-examined in the absence of fluorescence.

9. (403) When forwarding specimens or isolates to a reference laboratory, it is best to use

a. cotton-plugged tubes.	c. sealed petri dishes.
b. screw-capped tubes.	d. tightly sealed prescription bottles.

10. (404) The main advantage the slide culture has over other cultural methods is that it permits

a. the preparation of permanent mounts.
b. microscopic examination without disturbance.
c. a simple, rapid diagnosis.
d. the use of less strict aseptic technique.

11. (404) A disadvantage in the use of *scotch tape wet mount* is that it

a. destroys or damages the structural relationship of the fungus.
b. subjects the fungus to more trauma.
c. is more difficult to use.
d. cannot be preserved for future study.

12. (402) The fungi differ from bacteria in all of the following *except*

a. size.
b. nutrient requirements.
c. growth rate.
d. optimum pH requirement for growth.

13. (403) For which of the following diseases is there no isolation medium available at the present time?

a. Sporotrichosis.
b. Mycetoma.
c. Rhinosporidiosis.
d. Tinea nigra.

14. (404) The *India ink wet mount* technique is used primarily to detect *C. neoformans* in

a. spinal fluid.
b. all body fluids.
c. nocardiosis.
d. geotrichosis.

15. (404) The careful mycology worker should treat every specimen received in his department as if it contained

a. an avirulent agent.
b. a possible virulent agent.
c. a challenge to his diagnostic ability.
d. a highly virulent agent.

16. (404) The KOH wet mount is used to

a. detect the presence of fungal structures.
b. identify fungal agents.
c. determine if a culture is necessary.
d. eliminate saprophytic fungal structures.

17. (403) If fungal elements are *not* observed in a 10-percent KOH mount of clinical material, the specimen should be

a. reported as negative.
b. cultured on two tubes of Sabouraud's dextrose agar at both 25° C. and 37° C.
c. discarded aseptically.
d. cultured only at room temperature.

18. (405) Infections caused by the pathogenic yeastlike fungi are usually the result of

a. opportunistic hosts.
b. skin puncture.
c. inhalation of infective spores.
d. physiological changes in the host.

19. (404) Which of the following is *not* a growth characteristic of the fungi?

a. Structural relationship.
b. Tecture.
c. Surface topography.
d. Front pigmentation.

561

20. (403) Following direct examination of sputum for fungal structures, a Wright or Giemsa stain should be accomplished to aid in revealing the presence of

a. *Nocardia species.*
b. *Histoplasma capsulatum.*
c. *Cryptococcus neoformans.*
d. *Coccidioides immitis.*

21. (404) The techniques used in medical mycology are similar to those used in medical bateriology, except that in mycology more reliance is placed on

a. fermentation reactions.
b. serological tests.
c. colonial and microscopic morphology.
d. the isolation of pure colonies.

22. (402) Antibiotics may be incorporated into mycology media to inhibit contaminating

a. mycosis.
b. hyphae.
c. medically important fungi.
d. bacteria and saprophytic fungi.

Chapter 2

23. (411) Cervico-facial actinomycosis frequently develops as a result of

a. inhalation of infective spores.
b. tooth extraction.
c. blood stream dissemination.
d. working with cattle.

24. (405) A common procedure used to confirm a diagnosis of *Candida albicans* requires the demonstration of

a. chlamydospores.
b. arthrospores.
c. pseudomycelium.
d. true mycelium.

25. (406) An accurate diagnosis of tinea versicolor is dependent on

a. fluorescence under ultraviolet light.
b. direct microscopic examination in wet mount.
c. colony characteristics on artificial media.
d. both fluorescence under ultraviolet light and colony characteristics on artificial media.

26. (409) *E. floccosum* differs from the other dermatophytes by virtue of the fact that it will not invade the

a. nails.
b. skin.
c. hair.
d. subcutaneous tissue.

27. (410) Most infections with chromoblastomycosis are traceable to

a. inhalation of infective spores.
b. areas of the United States.
c. human to human exchange.
d. wounds contaminated with soil or vegetable matter.

28. (408) From the standpoint of disease severity, granular *T. mentagrophytes* differ from downy *T. mentagrophytes* in

a. being less virulent.
b. being more virulent.
c. being avirulent.
d. only in rate of growth.

591

29. (405) *Pityrosporum ovale* may best be differentiated from the other yeastlike fungi by its requirement for oleic acid and

a. the presence of dandruff.
b. its cream to tan colony color.
c. the presence of mycelium.
d. the presence of a cross-wall between the mother cell and the bud.

30. (407) The fungi which causes ringworm in dogs and cats as well as humans is called

a. *Microsporum canis.*
b. *Microsporum audouinii.*
c. *Microsporum gypseum.*
d. *Epidermophyton floccosum.*

31. (412) *Nocardia brasiliensis* is most often associated with

a. pulmonary nocardiosis.
b. mycetomal nocardiosis.
c. nocardiosis of the central nervous system.
d. penetrating thoracic nocardiosis.

32. (407) The dermatophytes generally limit themselves to the hair, skin, and nails because they

a. will not grow in the presence of Keratin.
b. will not grow in the absence of Keratin.
c. are classified as cutaneous fungi.
d. may be inhibited by substances in other body tissues.

33. (410) The sclerotic bodies formed in tissue by the various species of chromoblastomycotic fungi are

a. of less diagnostic value than serological tests.
b. all dissimilar.
c. all identical.
d. of less diagnostic value than animal inoculation tests.

34. (411) The most useful physiological characteristic of *Actinomyces israelii* and *Actinomyces bovis* in differentiation from saprophytic forms is their

a. pH requirement.
b. incubation temperature.
c. media requirement.
d. anaerobiasis.

35. (405) An identifying characteristic of *Geotrichum candidum* is its ability to

a. change its morphology as it matures.
b. cause colitis.
c. produce secondary infections.
d. cause mild respiratory infections.

36. (405) The microscopic finding of blastospores, pseudo, and true mycelium in wet preparation should suggest the presence of

a. *Trichosporon.*
b. *Geotrichum candidum.*
c. *Cryptococcus spp.*
d. *Candida albicans.*

37. (407) Tinea capitis is more prevalent in the United States among

a. boys.
b. girls.
c. animals.
d. adults.

38. (411) The members of the genera *Actinomyces, Nocardia*, and *Streptomyces* are all members of the class

a. *Ascomycetes.*
c. *Schizomycetes.*
b. *Eumycetes.*
d. *Deuteromycetes.*

39. (411) Clinical material in suspect cases of actinomycosis should always be searched carefully for

a. "sulfur granules."
c. blood.
b. pus.
d. clubs.

40. (405) Which of the following tests is *least* useful in identifying *Cryptococcus neoformans?*

a. Mouse pathogenicity.
c. Serological.
b. Urease.
d. Nitrate and sugar assimilation.

41. (410) Which of the following is *not* a type of sporulation demonstrated by the species causing chromoblastomycosis?

a. Acrotheca.
c. Disjunctor.
b. Phialophora.
d. Cladosporium.

42. (407) Wet mounts from cultures of *M. canis* display

a. many macroconidia and many microconidia.
c. few macroconidia and many microconidia.
b. many macroconidia and few microconidia.
d. few macroconidia and few microconidia.

43. (406) Cases of tinea nigra generally exhibit

a. a single lesion on the hand.
b. multiple lesions on the hand.
c. a single lesion on the other parts of the body.
d. multiple lesions on other parts of the body.

44. (405) The ability of *Cryptococcus spp.* to grow at 37° C. indicates that it is

a. definitely *C. neoformans.*
c. probably a saprophytic strain.
b. definitely a saprophytic strain.
d. possibly *C. neoformans.*

45. (412) Both *Nocardia spp.* and *M. tuberculosis* grow readily on media used for tubercular cultures; however, they may be differentiated on the basis of

a. incubation temperature.
c. Gram stain.
b. growth rate.
d. colonial morphology.

46. (407) The use of antibiotics in Sabouraud's dextrose agar when culturing the dermatophytes permits the worker to

a. decrease the pH to between 6.8 and 7.0.
c. elevate the pH to between 6.8 and 7.0.
b. incubate at 25° C. to 30° C.
d. elevate the pH to between 5.8 and 6.0.

47. (408) "Black dot" ringworm infections of the scalp are the result of a type of hair invasion called

a. endothrix.
c. ectothrix.
b. favic.
d. wedge perforation.

48. (405) The yeastlike fungus *Candida albicans* is usually classified as

a. superficial.
b. subcutaneous.
c. cutaneous.
d. systemic.

49. (409) The macroconidia of *Keratinomyces ajelloi* characteristically are

a. cylindro-fusiform.
b. spindle-shaped.
c. elliptically shaped.
d. ovate to pyriform in shape.

50. (406) Black piedra is a superficial disease of the

a. skin.
b. skin and hair.
c. hair.
d. mucous membranes.

51. (410) Mycetomas caused by the maduromycotic fungi may be distinguished from those caused by the *Actinomyces* and *Nocardia spp.* on the basis of hyphal diameter and

a. "grain" production.
b. clinical symptoms.
c. "grain" characteristics.
d. chlamydospore production.

52. (405) The yeastlike fungi which reproduce solely by simple budding and have the ability to encapsulate is called

a. *Candida.*
b. *Geotrichum.*
c. *Cryptococcus.*
d. *Trichosporon.*

Chapter 3

53. (414) Which of the following is most useful in establishing a confirmatory diagnosis of coccidioidomycosis?

a. Precipitin testing.
b. Animal inoculation.
c. Complement-fixation tests.
d. Each is equally useful.

54. (414) Which clinical form of North American blastomycosis most closely resembles the clinical picture seen in cases of sporotrichosis?

a. Disseminated.
b. Chronic intestinal.
c. Primary pulmonary.
d. Primary cutaneous.

55. (415) With reference to the mold form of *Histoplasma capsulatum,* the term "tuberculate" is used to describe the

a. macroconidia.
b. hyphae.
c. microconidia.
d. simple budding yeast cells.

56. (415) How should clinical material be examined for *Histoplasmosis capsulatum?*

a. In stained wet mounts.
b. In Wright or Giemsa stained smears.
c. In unstained wet mounts.
d. By all the above techniques.

57. (413) The yeast phase cells of *S. schenkii* are more easily observed when using

a. Wright or Giemsa stain.
b. the slide culture technique.
c. fluorescent antibody techniques.
d. unstained direct preparations.

58. (415) In order to confirm a cultural diagnosis of South American blastomycosis, it is essential to

a. perform animal inoculation tests.
b. demonstrate conversion capability.
c. perform complement-fixation tests.
d. perform intradermal skin tests.

59. (414) Which organism is the causitive agent of the disease sometimes referred to as "valley fever"?

a. *C. immitis.*
b. *S. schenckii.*
c. *C. neoformans.*
d. *H. capsulatum.*

60. (415) Which of the following is the *best* method for diagnosing infections of histoplasmosis?

a. Animal inoculation.
b. Cultural diagnosis.
c. Serological techniques.
d. Fluorescent antibody techniques.

61. (415) South American blastomycosis is caused by

a. *Paracoccidioides brasiliensis.*
b. *Coccidioides immitis.*
c. *Histoplasma capsulatum.*
d. *Blastomyces dermatitidis.*

62. (413) The parasitic or yeast phase of *Sporotrichum schenkii* produces cells best described as

a. arthrospores.
b. pyriform.
c. sporophores.
d. "cigar bodies."

63. (413) A dimorphic fungus is a

a. saprophyte at 37° C.
b. parasite at 25° C.
c. filamentous mold at 25° C.
d. mucoid yeast at 25° C.

64. (414) Addition of sterile normal saline to unopened culture tubes containing mycelial growth of systemic fungi is essential to

a. slow the growth rate.
b. retard aerosol formation.
c. prevent the media from drying out.
d. allow thorough absorption of nutrients.

65. (414) What is the most common manner of becoming infected with the systemic dimorphic fungi?

a. Inhalation.
b. Absorption.
c. Fomites.
d. Trauma.

66. (414) In which of the following areas is coccidioidomycosis endemic?

a. Southeast Asia.
b. Africa.
c. Southwestern United States.
d. Ohio Valley.

Chapter 4

67. (417) The branching brush-like conidiophore of the genus *Penicillium* is often referred to as

a. a sterigma.
b. a metula.
c. complex.
d. simple.

68. (416) Which of the following is absent in *Curvularia spp.?*

a. Dematiaceous hyphae.
b. Reverse pigment.
c. Unbranched conidiophores.
d. A cottony-type colony.

69. (416) The saprophyte *Sepedonium* closely resembles some strains of the genus

a. *Histoplasma capsulatum.*
b. *Blastomyces.*
c. *Coccidioides.*
d. *Paracoccidioides.*

70. (418) Laboratory identification of rhinosporidiosis is dependent on

a. direct examination of clinical material.
b. cultural studies.
c. clinical observation and histologic examination.
d. immunologic and animal inoculation tests.

71. (418) The most important clinical condition predisposing a phycomycotic infection is probably

a. diabetes mellitus.
b. prolonged use of antibiotics and steroids.
c. uremia.
d. malnutrition.

72. (418) The usual causative agent of pulmonary aspergillosis is

a. *Aspergillus niger.*
b. *Aspergillus amstelodami.*
c. *Aspergillus flavus.*
d. *Aspergillus fumigatus.*

73. (416) The genus name *Helminthosporium* has most probably originated as a result of the wormlike shape of the

a. conidia.
b. hyphae.
c. conidiophore.
d. colony.

74. (417) Which of the following saprophytes, having a characteristic thallus, first displays a pasty yeastlike colony which later becomes velvety and cottony?

a. *Rhodotorula spp.*
b. *Streptomyces spp.*
c. *Aureobasidium pullulans.*
d. *Geotrichum spp.*

75. (417) *A. fumigatus* is set apart from *A. clavatus* by its

a. flask-shaped vesicle.
b. growth ability.
c. unbranched chains.
d. smooth stalk.

76. (416) The macroconidia of *Alternaria spp.* have

a. no septations.
b. both transverse and longitudinal septations.
c. transverse septations only.
d. longitudinal septations only.

77. (418) *Cladosporium bantianum* may be differentiated from *Cladopsorium carrionii* on the basis of all of the following *except* the

a. growth rate.
b. length of spore chains.
c. development of longer conidia.
d. inability to liquify gelatin.

Chapter 5

78. (420) What part of the mature virus particle carries the genetic coding information for virus reproduction?

a. Nucleic acid core.
b. Capsid.
c. Envelope.
d. Ribosome.

79. (419) Which of the following recently developed techniques has simplified the isolation of virus cultures in the laboratory?

a. Atypical antibody levels.
b. Tissue culture methods.
c. Replicate plating.
d. Agar plate diffusion.

80. (421) Which one of the following members of the Psittacosis-Lymphogranuloma venereum-Trachoma (PLT) group causes a venereal disease that commonly affects the eyes of new-born infants?

a. Psittacosis agent.
b. Lymphogranuloma venereum agent.
c. Trachoma agent.
d. Inclusion conjunctivitis.

81. (421) The four groups of ribonucleic acid (RNA) viruses can generally be distinguished from the DNA viruses on the basis of symmetry of the protein in the coat, presence or absence of an envelope, the type of ribonucleic acid, and

a. inactivation by ether.
b. susceptibility to phage.
c. inactivation by monovalent cations.
d. microscopic morphology.

82. (420) The protein coat of the mature virus particle is known as the

a. capsomere.
b. capsid.
c. virion.
d. envelope.

83. (423) In the complement-fixation procedure, two systems are set up, i. e., the test system and the indicator system. Which of the following comprises the *indicator* system?

a. Serum, viral antigen, and complement.
b. Serum, sheep RBC, and hemolysin.
c. Sheep red blood cells and hemolysin (antibody to sheep RBC).
d. Complement, sheep RBC, and serum.

84. (422) In preserving clinical specimens for virus isolation attempts, which of the following factors is of greatest importance?

a. Selection of multiple specimens and protection from freezing.
b. Distance from the virus laboratory and time in transit.
c. An adequate clinical history and collection of paired sera.
d. Protection from drying and high temperatures.

85. (421) In collecting specimens for isolation attempts in a smallpox epidemic, you would take samples of which tissues?

a. Pustules, mucous secretions, and saliva.
b. Feces, urine, and saliva.
c. Pustules, blood, and urine.
d. Mucous secretions, liver biopsy material, and blood.

86. (419) Classification of viruses today is based on the presence or absence of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), along with other features. Of the four virus classification groups listed below, which one has RNA?

a. Adenoviruses.
b. Herpesviruses.
c. Papovaviruses.
d. Picornaviruses.

87. (423) If you mix dilutions of the patient's serum with a known virus and inoculate the mixture to eggs, tissue cultures, or animals, you are performing which virus diagnostic test?

a. Hemagglutination inhibition.
b. Complement fixation.
c. Neutralization.
d. Adsorption inhibition.

88. (422) Why is it necessary to obtain paired serum specimens 2 or 3 weeks apart in diagnosing a virus disease?

a. Virus isolations are made more easily as the disease progresses.
b. Antibodies are usually not formed during the early phase of the disease, and the first specimen provides a base line for the second in testing antibody levels.
c. The second specimen serves as a reserve supply of serum if the first becomes contaminated or is lost in transit.
d. Antibody titer drops rapidly after the first week of infection, and this decrease gives an indication of the patient's rate of recovery.

89. (423) A study of pathological changes in a mouse inoculated with a portion of a clinical specimen represents principally which approach to virus diagnosis?

a. Microscopic techniques.
b. Serological studies.
c. Virus cultivation.
d. Tissue (cell) culture.

90. (422) What constitutes a minimum set of specimens for the diagnosis of viral infections according to general guidelines suggested by authorities in the field of virology?

a. Skin scrapings, throat swab, and urine.
b. Throat swab or nasal washings, a stool specimen or rectal swab, and clotted blood.
c. Serum sample, urine, and throat washings.
d. A throat swab, a stool specimen, and a clinical history.

91. (423) Gel diffusion procedures in serology rely for success on what basis?

a. The gamma globulin fraction of the patient's serum can be coupled to fluorescent dyes without losing specificity for antigen.
b. The growth of tissue cells in a depression carved in an agar plate attracts bipolar antibodies from the agar.
c. A visible antigen-antibody reaction results from the diffusion of antigen and antibody through a solid menstrum.
d. Pooled Group O blood cells agglutinate when virus particles grown in tissue culture are adsorbed onto these cells.

92. (420) During virus replication, protein components and nucleic acid elements for newly manufactured virus particles are assembled by

a. extracellular exoenzymes.
b. neighboring cells.
c. random spontaneous polymerization.
d. the host cell.

93. (419) According to *Bergey's Manual* on classification of microorganisms (7th Edition), which two orders fall within the class *Microtatobiotes*, the rickettsial and viral organisms?

a. *Virales* and *Rickettsiales.*
b. *Virales* and *Chlamydiaceae.*
c. *Miyagawanella* and *Rickettsiales.*
d. *Chlamydia* and *Coxiella.*

94. (421) The *Papovavirus* group of DNA viruses gains its name from a virus common to

a. mice only.
b. monkeys only.
c. man and rabbits.
d. mice and rabbits.

95. (422) To preserve clinical specimens for a period of a few hours prior to shipment, what means of preservation would you choose?

a. Store at room temperature.
b. Freeze specimens at -70° C.
c. Add an equal volume of 50 percent glycerin.
d. Store specimens at 4° C.

96. (420) Which of the following statements is accurate?

a. Viruses are facultative parasites.
b. Viruses are facultative saprophytes.
c. Viruses are obligate parasites.
d. Viruses are obligate saprophytes.

97. (421) Which one of the following statements about the Arboviruses is *not* correct?

a. Some arthropod-borne viruses multiply in man or animals as well as in their primary blood-sucking hosts.
b. A viremia (viruses in the blood stream) is apparently necessary for natural transmission by arthropods, but in some instances secondary infections in animals can arise from contact between animals.
c. Arboviruses contain RNA but not DNA.
d. Group A viruses, represented by Eastern, Western, and Venezuelen encephalitis, are transmitted by mosquitoes, ticks, and certain species of biting flies.

98. (420) What are the two symmetries or configurations into which virus protein molecules are generally arranged?

a. Helical and trapezoidal.
b. Rhombic and trapezoidal.
c. Cubic and helical.
d. Cubic and rhombic.

Foldouts 1 thru 3 of this publication have been deleted in adapting this material for inclusion in the "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education." These foldouts contained color photographs which could not be reproduced.